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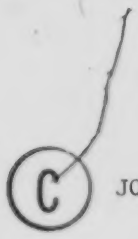

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled MEMBRANE LIPID PROPERTIES AND MEMBRANE FUNCTION: SOME STUDIES AT THE PHYSICAL-BIOLOGICAL INTERFACE submitted by JOHN R. SILVIUS in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

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Nel mezzo del cammin di nostra vita

Mi ritrovai per una selva oscura

Che la diritta via era smarrita...

Dante Alighieri

ABSTRACT

The aims of this work were twofold: first, to generate a biological membrane system in which the lipid fatty acyl composition can be varied essentially at will, and to study the relationship of lipid properties to membrane function in such a system, and secondly, to synthesize and characterize some novel phospholipids which provide valuable model systems whose behavior is relevant to that of the lipids in the biological membrane studied.

Toward the former objective, I have developed a simple method for converting the simple cell wall-less prokaryote Acholeplasma laidlawii B into a total fatty acid auxotroph, using avidin to completely inhibit de novo fatty acid synthesis and chain elongation in vivo. Cells thus treated can grow well when any of a wide variety of fatty acids are added to the culture medium, and if a single fatty acid is added, the cells incorporate it into their membrane lipids to the extent of 95+% of the total lipid acyl chains if the fatty acid can support cell growth. The cellular growth-supporting ability of a fatty acid appears to rest on its physical and not its metabolic properties. 'Fatty acid-homogeneous' membranes or membrane lipids, obtained from cells grown with avidin plus any of a number of single fatty acids which are good growth substrates, exhibit a markedly sharper lipid phase transition by differential thermal analysis than do normal membranes or membrane lipids, which have a heterogeneous fatty acyl composition. The protein and lipid head-group composition of fatty acid-homogeneous membranes varies somewhat depending on the nature of the lipid acyl chains, but a variety of experiments indicate that such variations are unlikely to produce changes of a

qualitative nature in the physical behavior of the membrane lipids. The potential for manipulation of lipid fatty acyl chains and physical properties in the A. laidlawii B membrane has been used to study the physical basis of the membrane lipid effects on the temperature dependence of a membrane enzyme activity, the (Na^+,Mg^{2+})-ATPase, which appears to function as an osmoregulatory cation pump in vivo. My results, analyzed in the light of an extensive theoretical analysis of Arrhenius plot behavior, suggest that the ATPase's catalytic activity is not altered by changes in the lipids around it so long as they are in the liquid-crystalline state, but that a membrane lipid phase transition occurring on cooling the membrane can cause the boundary lipids of the ATPase to enter a 'gel-like' state and thereby to inactivate the enzyme.

Working toward the second objective noted above, that of developing and characterizing good pure-lipid model systems for the fatty acid-homogeneous A. laidlawii B membrane, I synthesized a number of branched-chain, odd-chain saturated, monounsaturated and cyclopropane fatty acids and their phosphatidylcholine (PC) derivatives. I then characterized the thermotropic behavior of the fully hydrated PC's by differential thermal analysis. By studying the effect of acyl chain length and structure on the thermotropic behavior of hydrated diacyl PC's, I have been able to draw various conclusions regarding the nature of the acyl chain packing in the gel state and regarding the function of various types of methyl-branched and alicyclic lipid acyl chains in membranes. Finally, I have shown that the gel-to-liquid-crystalline phase transition temperatures of pure PC's and of A. laidlawii B membrane lipids of like fatty acyl composition correlate very well, indicating that the lipid acyl chain interactions in the two systems are fundamentally similar and that

the former lipid system is in fact a good model for the biological membrane.

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ABBREVIATIONS AND SYMBOLS USED IN THE TEXT

(Certain nonstandard symbols used only in one section of the text are defined in the appropriate sections and are not listed below. The shorthand nomenclature for fatty acids used in this text is described in Appendix 1.)

A_x	absorbance at x nanometers
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ATPase	ATP β,γ-phosphohydrolase
°C	degrees centigrade
$\Delta C_p^{\ddagger}$	heat capacity of activation
DCCD	dicyclohexylcarbodiimide
DGDG	diglucosyl diglyceride
DSC	differential scanning calorimetry
DTA	differential thermal analysis
EDTA	ethylenediaminetetraacetic acid disodium salt
ESR	electron spin resonance
GLC	gas-liquid chromatography
g	gram
GPDGDG	glycerophosphoryl diglucosyl diglyceride
ΔH	enthalpy change
$\Delta H^{\ddagger}$	activation enthalpy
°K	degrees Kelvin (absolute temperature)
kcal	kilocalorie
K_m	Michaelis constant

l or ℓ	litre
M	molar
m-, μ-, n-	milli-, micro-, nano- (10^{-3}, 10^{-6}, 10^{-9})
min	minute
MsCl	methanesulfonyl chloride
n	carbon number
NEM	N-ethylmaleimide
NMR	nuclear magnetic resonance
O.D.	optical density (absorbance)
O-PG	O-aminoacyl phosphatidylglycerol
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PCMBS	p-chloromercuribenzenesulfonate
PE	phosphatidylethanolamine
P_i	inorganic phosphate
PG	phosphatidylglycerol
R	gas constant (1.987 cal $mole^{-1}$ deg^{-1})
$\Delta S^{\ddagger}$	activation entropy
SDS	sodium dodecyl sulfate
T	temperature
t	time
T_{AL}	midpoint of A. laidlawii B membrane phase transition
T_c	lipid gel-to-liquid-crystalline transition temperature
TEA	triethylamine
TLC	thin-layer chromatography
T_m	melting temperature
V_{max}	maximal velocity of enzymic reaction

CHAPTER 1

INTRODUCTION

The determination of the equilibrium or time-averaged molecular structures of biological assemblies has long been, and continues to be, a major emphasis of contemporary research in molecular biology. A new area of interest, which a number of researchers are beginning to explore, is the study of the dynamic (i.e., time-dependent) aspects of molecular and supramolecular structure. The reason for this current interest in molecular dynamics is twofold. First, a static, precise spatial description of a system's 'structure' is actually an average over many instantaneous configurations and as such gives a complete description of the system's state only when information about the rates and amplitudes of fluctuations about this average 'structure' (i.e., the dynamic properties of the system) is added to it. Secondly, almost all of the biologically important functions of macro- or supramolecular systems are time-dependent and therefore have their basis in the dynamic properties of such systems. Just as the structures of large molecular assemblies can be related to the structures of their constituent molecules, so the time-dependent functions of the assemblies can be related to the temporal fluctuations in the states of their molecular components. Therefore, a knowledge of molecular dynamics is required both to fully define the 'state' of a system (i.e., its structure over time as well as over space) and to analyze the molecular origins of the system's dynamic properties. The addition of the dimension of molecular dynamics to more classical ideas of 'structure' promises to transform our ways of conceptualizing and investigating the nature of biochemical systems, and nowhere is this

more true than in the field of membrane biology. The development of powerful yet adaptable experimental techniques for the study of molecular dynamics in membranes, and of complementary experimental systems which are at once relevant to biological systems and amenable to sophisticated physical studies, is one of the major challenges facing membrane workers today. It is this challenge, and some current efforts to meet it, that shall form our primary interest and primary objective in this thesis and in the work to be described in it.

Any study of fluctuations of molecular arrangements about the 'average' structure must begin by establishing what is the nature of this 'average'. In the case of membranes, recent work has largely resolved this latter question, as the following short review will indicate.

After many years of debate, certain basic aspects of membrane equilibrium structure are now widely agreed upon. The lipids and proteins in most membranes appear to be arranged in an amphipathic lamellar structure, with a rather nonpolar, almost anhydrous inner layer flanked by two layers of more polar, hydrated structures which are in contact with the aqueous phase. The apolar layer contains the fatty acyl chains of the membrane lipids (which are arranged in a bilayer), the hydrocarbon moieties of sterols or isoprenols, and many of the hydrophobic, but few of the hydrophilic, amino acid residues of membrane proteins. By contrast, the surface layers contain the lipid phosphate ester groups, sugar and amino acid residues of lipids and proteins, and substantial quantities of water and inorganic ions (for reviews, see Singer and Nicolson, 1972; Bretscher, 1973; Singer, 1974; Gulik-Krzywicki, 1975). If we neglect the transverse asymmetry of many membrane components (Rothman and Lenard, 1977; Bergelson and Barsukov, 1977), the basic amphipathic lamellar

structure of membranes seems to be an equilibrium configuration of the highly amphipathic molecules that comprise them. Most membrane lipids, along with many membrane proteins, when isolated and reconstituted in excess water will spontaneously form lamellar bilayer structures very similar to those found in the membranes from which they were derived (Bangham et al., 1974).

Most of the lipids, and many of the protein components of membranes, do not appear to be strongly ordered or motionally hindered within the plane of the membrane (Edidin, 1974). This finding has led to the currently popular concept of the membrane as a two-dimensional solution in which protein and lipid molecules can diffuse fairly freely in directions parallel to the membrane surface (Singer and Nicolson, 1972). The lipids, being smaller and more plastic than the proteins, can be considered to be the 'solvent' in such a system, and for this reason considerable attention has been paid to the influence of lipid properties on the structure and function of the membrane as a whole. Within a biologically relevant range of temperatures (roughly -5°C to +80°C), most membrane lipids form bilayers in excess water and can exist in two states: a gel state, in which the acyl chains adopt an extended, all-trans-methylene configuration and are arranged strictly in parallel arrays (Gulik-Krzywicki, 1975), and a liquid-crystalline state, in which the acyl chains are still highly ordered near their attachments to the lipid glycerol or sphingosine backbone but are quite disoriented and rapidly mobile near their methyl termini, exhibiting a so-called 'fluidity gradient' (Hubbell and McConnell, 1971; Seelig and Seelig, 1974; Lee et al., 1976). The transition between these states in pure lipid-water systems has been extensively investigated; it is moderately cooperative (i.e., involves the coupled,

simultaneous melting of domains extending over tens or hundreds of lipid molecules) and is thought by many workers to be a typical first-order transition (Mabrey and Sturtevant, 1976; Lee, 1977). This phase transition can be induced by changes in temperature (thermotropic), in the water content of the system (lyotropic) or in the ionic content of the medium (ionotropic), and the transition in excess water (which is the biologically relevant state) can be influenced by divalent cations, proteins, sterols, and a number of lipophilic compounds of pharmacological importance (Seeman, 1972; Trauble and Eibl, 1974; Lee, 1977a,b). As a consequence of these influences, and of the heterogeneous lipid composition of biological systems, the lipid phase transition in natural membranes is often broad (usually some tens of degrees wide). Interestingly, thermotropic changes in lipid properties are often observed just below or within the range of acceptable growth temperatures for a variety of organisms, suggesting that such changes may be of biological importance (McElhaney, 1974; Cronan and Gelmann, 1975).

While the properties of the membrane lipid 'solvent' have been extensively studied (and are, in some cases, understood), less is known about the structure and lipid 'solvation' of membrane proteins, although some useful basic principles are beginning to emerge from recent work. Membrane protein structure appears to conform rather closely to the demands of its highly anisotropic, inhomogeneous lipid 'solvent', burying polar functions and exposing hydrophobic amino acid side-chains in the membrane's apolar layer while exhibiting more conventional features in the polar surface layers (Capaldi, 1977; Guidotti, 1977; Singer, 1977). Membrane proteins may preferentially associate with certain lipid classes (e.g., anionic lipids (Boggs et al., 1977a,b; Jost and Griffith, 1978))

and may require such lipids for proper function (Sandermann, 1978; De Pont et al., 1978). The 'boundary' lipid in contact with 'integral' membrane proteins (loosely, those proteins which penetrate into the a-polar layer and are not simply adsorbed to the polar surface) appears to differ in its properties from the 'bulk' lipid (most notably, it is less mobile (Jost et al., 1973)), although the exact extent and nature of the difference is controversial.

With these findings about membrane 'structure' in mind, we now return to the problem of studying membrane dynamics. In contrast to the advances just described in the study of equilibrium structures in membranes, the dyanmic properties of membrane molecules are much more poorly understood. For example, no experimentally verified molecular model for transmembrane substrate transport by a membrane carrier protein has appeared to date in the literature, yet the structure of a membrane proton transporter, the purple membrane protein of Halobacterium halobium, has been determined to a resolution of roughly 7 Å by electron diffraction (Unwin and Henderson, 1975; Henderson and Unwin, 1975). This lack of information regarding molecular dynamics is a serious obstacle to our understanding of membrane function, for an appreciation of the transient features of membrane structure is essential to an understanding of transport, enzyme activities, regulatory processes and a host of other time-dependent phenomena that occur in membranes.

One very important part of the study of membrane molecular fluctuations is the analysis of the dynamic properties of membrane lipids and of their influence on the functional properties of membranes. In this study, we shall be concerned mainly with the development of suitable methodologies for the study of this latter aspect of membrane properties.

Such methodologies may also, of course, aid more conventional 'structural' studies. As we shall see, both the physical techniques used for the study of membranes and membrane lipids, and the model systems from which basic physical information about lipid properties and functions is derived, will need to achieve a greater level of sophistication in order to furnish useful, rigorous results in the area of biological membrane lipid dynamics.

Two types of studies can furnish useful information regarding lipid dynamic properties and their effects on membrane function. The first type of study monitors the behavior of the lipids themselves and usually requires the introduction of some sort of probe molecule to achieve a suitable signal strength or selectivity for detailed interpretation of the results. This type of study is limited at present by the ability of the experimenter to rigorously interpret the complex results normally obtained, since the molecular motions of interest are generally highly anisotropic and are characterized by multiple correlation times or time constants. Such experiments often require large amounts of physical data or extensive simulation studies in order to extract the lipid motional or conformational properties from the data. As well. it is always important in such experiments to distinguish what aspects of the lipid probe's behavior are truly representative of the lipid behavior and what aspects are simply artefacts unique to that probe. The second approach to study membrane lipid effects on membrane dynamic properties observes the lipids indirectly, monitoring some characteristic function of the membrane (e.g., glucose transport) under different conditions in which the lipid physical properties vary. In membrane lipid studies, the lipid properties are usually altered by manipulating the temperature and the

lipid composition and monitoring the effect of the lipid phase transition on the process of interest (Cronan and Gelmann, 1975; Linden and Fox, 1975). This approach has been useful because the differences between gel-state and liquid-crystalline lipids are very substantial and have been fairly well defined, at least qualitatively, by a variety of experimental approaches (Ladbrooke and Chapman, 1969; Lentz et al., 1976; Neiderberger and Seelig, 1976; Gent and Ho, 1978; Gaber et al., 1978a). When the differences in the lipid composition and/or physical properties are more subtle, little basic information is available for use in interpreting the experimental results, which must therefore be discussed on a largely phenomenological basis. The challenge of inducing membrane lipid changes which are of sufficient magnitude to allow ready interpretation of their effects in terms of model-system physical results, yet which are not so great as to force the system past the point of normal biological function, is an immense one and has not often been successfully met.

The study of membrane lipid dynamics and of their effects on membrane function is therefore limited by two factors: the ability of physical techniques to observe and define the lipid behavior, and the ability of the investigator to manipulate the composition and physical properties of the lipids in a systematic, well-defined manner. As a survey of available techniques for monitoring lipid dynamics and for manipulating lipid composition in various systems will reveal, these limitations have become progressively less severe over the last few years. As we shall see, it should be possible in principle to obtain high-quality analyses of lipid dynamic properties in membranes, and of their role in various membrane processes, so long as proper efforts are devoted to developing suitable membrane model systems for this purpose. To understand what

constitutes a 'suitable' membrane system, we must consider the types of physical methods available for probing membrane lipid dynamics.

The motions of membrane lipids typically exhibit correlation times (which roughly correspond to the average time required for rotation through a given angle or translation through a given distance) in the range of 10^{-10} sec (for single-bond rotations and vibrations) to several seconds (for lateral diffusion of integral membrane proteins). Since the techniques used to observe these motions must usually possess a temporal resolution greater than or roughly equal to the time constants for the processes of interest, it is not surprising that spectroscopic methods have proven most useful in this area of membrane research. Other techniques, such as differential scanning calorimetry (DSC) (Ladbrooke and Chapman, 1969; Melchior and Steim, 1976) and X-ray diffraction (Engelman, 1971) are useful for monitoring lipid phase transitions but furnish little useful information regarding the motional properties of liquid-crystalline lipids. Three types of spectroscopy have proven very useful for studies of membrane lipid dynamics: magnetic resonance, vibrational spectroscopy and fluorescence relaxation techniques. Let us briefly consider the capabilities and limitations of each technique in turn.

In magnetic resonance experiments, a static magnetic field splits the energies of the various spin states of an electron or certain atomic nuclei (hereafter called simply a 'spin'), and the interstate transitions of the individual spins sum to give net changes in the bulk magnetization of the sample when a fluctuating transverse magnetic field perturbs the system from equilibrium (Lee et al., 1974; James, 1975). The relaxation of the bulk magnetization after such a perturbation is characterized by

two time constants: T_1, the rate of transition between spin states, and T_2, the rate of relaxation of the net transverse magnetization induced by the fluctuating field. For certain nuclei of interest to membrane biologists (^1H and ^{13}C, for example), both T_1 and T_2 are sensitive to molecular motions. Under favorable circumstances, one can determine the dominant contributions to the magnetic relaxations and deduce the time constants for one or more types of molecular motions. If simple T_1 and T_2 measurements are supplemented with the results of more sophisticated experiments (multiple-irradiation experiments, for example (James, 1975; Yeagle et al., 1977)), a considerable amount of information about molecular motion in membranes can be obtained. Certain spins (unpaired nitroxide electrons, ^2H, ^{19}F and ^{31}P, for example) can also furnish more or less readily interpretable information regarding molecular orientation and the anisotropy of molecular motion in oriented or unoriented bilayer systems.

Along with these capabilities, magnetic resonance studies have one major drawback: they furnish a great deal of information compressed into a single linewidth or T_1 value. The great challenge in such studies is to break down the overall spin relaxation processes into their various contributing mechanisms. The difficulty of this task is well exemplified in the case of the nitroxide spin labels, whose spectra in membranes were long rendered readily interpretable only through the use of a model which is now thought to be invalid (Mason and Polnaszek, 1978). If one considers this difficulty, plus the considerable controversy surrounding the question of the extent of perturbation of a spin label probe's environment by the probe moiety's bulky structure (Cadenhead and Müller-Landau, 1975), it may seem rather surprising that membrane workers

continue to employ spin labels in their studies. The explanation for the popularity of this method is, however, simple enough: ESR is greatly more sensitive than is NMR, requiring only about one one-thousandth of the spins needed to generate a macroscopically detectable nuclear magnetization, and small samples, or samples with limited numbers of sites to be reported by the probe, have thus generally been the natural preserve of the ESR technique. Recently, however, the advent of signal-averaging Fourier-transform NMR (Lee et al., 1974; James, 1975), and of detection systems capable of collecting a larger portion of the total spin relaxation than has previously been possible, has enhanced the potential sensitivity of the NMR technique to a point where sample size need not be a primary factor in choosing the type of probe to be used in magnetic resonance experiments. The ideal magnetic resonance technique for membrane lipid studies would be sensitive enough to detect a signal from a few milligrams of membrane and selective enough to allow a one-to-one assignment of the resonances detected to the various chemically distinct spins generating them. It would also use normal membrane lipids, or compounds of essentially identical structure, as probe molecules, would provide information on both molecular order and molecular motion, and would allow the ready chemical synthesis of appropriate probes where necessary. The closest approaches to this ideal at present are ^{2}H quadrupole resonance and ^{19}F-NMR, but ^{13}C- and ^{31}P-NMR will also prove useful in certain cases.

While magnetic resonance methods in the solid state probe molecular motions and orientations, vibrational spectroscopy probes the structure and conformation of molecules. The basis of the utility of vibrational spectroscopy (i.e., infrared and Raman spectroscopy) in studies of mole-

cular conformation is the dependence of vibrational transition frequencies and probabilities on the distribution of the resonating atoms in space and on the bonding energies among the various atoms. As certain vibrational modes may involve a large number of nuclei (the 'acoustic-band' resonance of all-trans polymethylene chains is one such example), it is possible to examine the conformations of large segments of the molecule(s) of interest by measuring the frequencies and intensities of molecular infrared absorbance or Raman scattering (Yellin and Levin, 1977; Gaber et al., 1977; Koyama et al., 1977). Lasar-Raman scattering methods have recently become popular for studies of simple lipid or lipid-protein systems, providing an ensemble average of the instantaneous conformations of the molecules. Considerable effort has been devoted to assigning the various frequency bands to various vibrational modes, and on this basis the distribution of all-trans segments, of gauche conformations, and of 'kinks' ($g^+ t g^-$ conformations (Träuble, 1971)) in the lipid methylene chains have been estimated (Yellin and Levin, 1977; Gaber et al., 1977). Wallach and his coworkers (Verma et al., 1975; Verma and Wallach, 1976) have used Raman scattering effects to detect a thermotropic change in the structure of the membranes of erythrocytes and lymphocytes. The great advantage of the lasar-Raman technique is that it can directly furnish information regarding the transient conformations of structural units in the lipid molecules which are larger than single methylene segments or phosphate groups. The disadvantages of the method include a lack of information on the lifetimes of the molecular conformations (it is a 'snapshot' technique) and the considerable overlap of certain frequency bands of interest with adjacent bands (although this latter problem may sometimes be circumvented by the use of deuterated

lipids (Gaber et al., 1978b; Mendelsohn and Maisano, 1978).

A third useful method for investigating membrane lipid motions, fluorescence spectroscopy, gives results which largely reflect the reorientation and diffusion of the probe molecules. Since few types of molecules in membranes are strongly fluorescent (tryptophan is one notable exception), it is usually necessary to incorporate an extrinsic probe into the membrane in fluorescence studies. Fluorescent lipid probes are of three types: lipophilic or amphipathic molecules largely unlike natural lipids in structure, such as 1,6-diphenyl-1,3,5-hexatriene (DPH) and 8-anilinonaphthalene-1-sulfonic acid (ANSA) (Jacobson and Papahadjopoulos, 1976; Andrich and Vanderkooi, 1976); natural lipids with an unnatural fluorescent moiety attached, such as the anthroylstearic acid probes (Cadenhead et al., 1977); and conjugated polyene fatty acids, such as α- and β-parinaric acids and their phospholipid derivatives (Sklar et al., 1975, 1976). While fluorescence intensity measurements can be used to monitor lipid phase transitions, such measurements are of little use in determining the absolute values of any useful motional parameters for the membrane lipids. Fluorescence depolarization measurements, by contrast, can in principle furnish considerably more useful data (Shinitzky and Barenholz, 1978). Since the probability of excitation of a fluorescent probe by a polarized beam of light is maximal when the electric vector of the beam is parallel to the molecular transition moment, the population of molecules excited by a rapid pulse of polarized light will have a net preferential orientation along the axis of polarization of the light beam. This net orientation of the excited molecules will decay to some extent, due to random molecular motions, between the time of absorption and the time of emission of the exciting photons; the

beam of emitted photons will therefore be less polarized the greater the extent of molecular tumbling during the lifetime of the excited state. If a satisfactory model for the molecular tumbling is available, and if the excited-state lifetime is known, a rotational correlation time (or times, if the motion is anisotropic) can be calculated and can in some cases be related to a membrane 'microviscosity'. In general, however, the motional and viscosity parameters extracted from fluorescence depolarization experiments are highly model-dependent (Kinosita et al., 1977; Chen et al., 1977; Dale et al., 1977). An adequate model of probe motion is probably more readily derivable for a highly elongated, roughly axially symmetric probe, such as a parinaric acid (which is likely to align itself parallel to the lipid acyl chains), than for an irregularly shaped probe such as N-phenylnaphthylamine (NPN), whose motions and orientation are much more difficult to predict. More direct measurements of lateral (as opposed to rotational) diffusion rates are possible using fluorescence correlation spectroscopy (FCS) or fluorescence recovery after photobleaching (FRAP) (Axelrod et al., 1976; Koppel et al., 1976; Smith and McConnell, 1978), which measure the temporal fluctuations of probe surface density or the rate of recovery of the equilibrium probe surface density after rapid local depletion by a high-intensity light flash. FCS and FRAP will probably prove to be the most useful fluorescence methods for obtaining quantitative information on the motional properties of membrane molecules, but even in this case, the preparation of suitable, reliable fluorescent lipid probes may pose a major difficulty.

This brief overview of the available physical methods for monitoring membrane lipid dynamics should provide ample evidence to support the conclusion that no ideal membrane lipid probe technique currently exists.

While this fact alone is scarcely cause for despair, there is a more fundamental problem which is common to all of these probe techniques and which may limit their potential for high-resolution studies in membranes. This latter problem stems from the complicating effects on measured probe signals that arise when multiple environments are present in the system of interest. Unless the probe employed is extremely selective for a single environment or gives readily distinguishable, resolvable signals when present in each of the different environments, the spectroscopic signal that the probe generates will represent a complex summation of signals from various local domains. In this latter case, the decomposition of the overall signal into its components, or even the extraction of an 'average' parameter which has some physical meaning at the molecular level, may be difficult if not impossible. This problem is matched by the equally serious possibility that a probe may report one environment in preference to the others present in a membrane system. If this selectivity is not adequately accounted for, serious errors in experimental interpretation can result. From the above considerations, it can be appreciated that the currently available or foreseeable physical methods for probing membrane lipid dynamics can be most useful (i.e., can yield the most reliable data) when the membrane lipid domain is as close to being a single, homogeneous phase as proper membrane function will permit.

Since microenvironmental homogeneity usually requires homogeneity in the chemical composition of a molecular ensemble, it is not surprising that many workers, wishing to avoid the problem of multiple microenvironments in the lipid system under study, have carried out a great number of physical studies on pure dipalmitoyl phosphatidylcholine in aqueous

dispersions. Unfortunately, a direct extropolation from such a system to, for example, a HeLa cell membrane is, at best, tenuous. A more useful membrane model would combine a simple lipid composition with a demonstrable biological activity. Three major approaches have been used to obtain systems of this latter type: membrane reconstitution, *in vitro* modification of membrane lipid, and *in vivo* (metabolic) manipulation of lipid composition. As the following brief review will demonstrate, each approach has its own strengths and limitations.

The most satisfactory method for obtaining a membrane-like system of very simple composition is to solubilize and then isolate a single protein or protein complex from the membrane, using any of a variety of treatments to weaken the interactions of the membrane components with one another, and then to reconstitute the isolated protein with a single bilayer-forming lipid. This approach has proven quite successful for several proteins, including notably the Ca^{2+}-ATPase of sarcoplasmic reticulum (Hesketh *et al.*, 1976), the (Mg^{2+},Na^{+},K^{+})-ATPase of eukaryotic cell membranes (Hilden and Hokin, 1976), cytochrome b_5 of microsomal membranes (Dufourcq *et al.*, 1976), and the mitochondrial cytochrome oxidase (Vik and Capaldi, 1977). While reconstitution studies are likely to constitute an important branch of membrane research for some years to come, they suffer from certain basic drawbacks. First, the tertiary structure of the reconstituted protein may no longer be the same as that of the native membrane-bound species; a number of recent studies have demonstrated that protein structure can be quite plastic even under conditions usually assumed to be 'benign' (Clarke, 1977; Nozaki *et al.*, 1978). An alteration of enzyme activity or regulatory properties in a reconstituted system cannot automatically be attributed to the effect of the lipid

environment, for rigorous controls are necessary to ensure that the protein structure has not been irreversibly altered during solubilization and reconstitution. Secondly, the composition of the reconstituted system may be made so simple that certain accessory elements that influence the system's function *in vivo* may be omitted. Again, the simple retention of the gross properties of the system when reconstituted does not guarantee that its structure is native in all respects. Thirdly, it is often difficult to ensure complete removal of the membrane-disrupting agent from the reconstituted system and thus to avoid undesirable side effects of the disrupting agent. For example, residual sodium deoxycholate in a reconstituted (Mg^{2+},Na^{+},K^{+})-ATPase apparently abolishes the anionic lipid dependence of this enzyme (Mandersloot *et al.*, 1978). Finally, many interesting but complex cell functions (e.g., growth or differentiation) are simply impossible to transfer to a reconstituted system, at least at present. In spite of these drawbacks, the reconstitution method remains the only currently available general method by which lipid-protein interactions, and their functional consequences, can be studied at high resolution.

If homogeneity of protein composition is not required in a study of membrane dynamic properties, it is sometimes possible to alter, and in some cases to simplify, the lipid composition of the membrane *in vitro* without disaggregating it. Among the methods by which such alterations may be achieved are phospholipase treatments (Roelofsen and Zwaal, 1976; Bevers *et al.*, 1978; De Pont *et al.*, 1978), phospholipid or sterol exchange (Zilversmit and Hughes, 1976; Deuticke and Ruska, 1976; Kahane and Razin, 1977), and membrane fusion (Houslay *et al.*, 1976; Poste and Papahadjopoulos, 1976). Phospholipase degradation of specific lipids

has been used to demonstrate a specific lipid requirement for several membrane enzymes such as the eukaryotic (Mg^{2+},Na^{+},K^{+})-ATPase (De Pont et al., 1978). Certain readily available lipolytic enzymes, such as phospholipase D and phosphatidylserine decarboxylase, can convert one diacyl phospholipid species to another without further degradation to membrane-disrupting species such as lysolipids. The potential for use of phospholipases to modify membrane lipids is limited, however, for many phospholipolytic enzymes do produce membrane-disrupting species or require some form of membrane perturbation (such as cellular lysis or the use of detergents) in order to modify a significant fraction of the lipids. Phospholipid or sterol exchange or membrane-vesicle fusion can simplify the membrane lipid composition only if the reservoir of lipids in the donor system is very large, so that the intrinsic lipids of the acceptor membrane are diluted to negligible levels. To date, the transfer efficiency of these processes has generally proven too low to accomplish major lipid replacements in samples of appreciable size, although this is a technical problem rather than a fundamental limitation of such procedures. More serious are the problems of wholly separating the lipid donor and acceptor systems after interaction has occurred, and the possibility of undesirable intervesicular transfer of membrane components other than lipids (Enuch et al., 1977). It appears at present that in vitro lipid modification methods will prove useful for altering the lipid head-group and the sterol composition of the membrane but will be of little use for simplifying the membrane lipid composition or for drastically altering the lipid fatty acid composition.

The third major method for manipulating membrane lipid composition is the in vivo manipulation of the lipid metabolism, and thereby the

lipid composition, of a living organism. A net simplification of the cellular lipid composition is achieved when synthesis of a nonessential lipid species is inhibited or when a pathway generating a variety of lipid species is inhibited and a single exogenous species is used to replace the normally heterogeneous complement of endogenously synthesized species. Relatively few studies have appeared in which the synthesis of a particular phospholipid or sterol has been inhibited without substituting a lipid species of related structure in order to maintain cell viability (see, however, Astin and Haslam, 1977; Ohta and Shibuya, 1977; Chen et al., 1978). It has been possible in some systems to largely or wholly inhibit endogenous fatty acid synthesis and to replace at least a portion of the fatty acids normally found in the membrane lipids with a single exogenous acyl species. This technique has been most successful in simplifying the fatty acid composition of certain microorganisms, notably Escherichia coli (Baldassare et al., 1976, 1977), Saccharomyces cerevisiae (Astin et al., 1977) and Mycoplasma strain Y (Rodwell, 1968; Rodwell and Peterson, 1971). Recent results with cultured mammalian cell lines have also indicated a considerable potential for simplification of lipid fatty acyl composition by in vivo modifications in these systems (Horwitz et al., 1978). Enrichments of membrane lipids in a single fatty acid species to the extent of > 90% of the total acyl chains have been achieved, using a limited number of exogenous fatty acids, in E. coli and Mycoplasma strain Y, although the lipids of the former organism can be highly enriched in only a few cis-monounsaturated fatty acids and the latter organism requires large amounts of membrane cholesterol for growth. In spite of their limitations, these advances illustrate the potential of in vivo lipid modification methods for generating membranes

whose lipid composition is simple enough to be amenable to rather sophisticated physical studies of membrane lipid properties.

The preceding survey of available experimental methods for probing membrane lipid dynamics and for modifying membrane lipid composition leads to the conclusion that at present there is no physical technique which is perfectly suited to the study of biological membranes and no natural membrane system which is perfectly suited to examination by physical techniques. Unfortunately, this problem has led many physical biochemists to study pure lipid-water systems instead of membranes, while many more biologically oriented workers who apply physical techniques to membranes produce very qualitative and uncritical interpretations of the data obtained. It would clearly be helpful to membrane workers to have as a model system a biological membrane whose lipid composition is sufficiently simple and readily manipulable to allow the properties of the lipids to be examined in detail by physical methods. The information derived from studies on such a system, combined when necessary with information from studies of the isolated lipids in aqueous dispersion or reconstituted with membrane proteins, could furnish considerable information, not only on the dynamic properties of membrane lipids, but also on the utility and the weaknesses of various physical probes of lipid properties when applied to biological membranes. Such information would be of incalculable value in interpreting the results of studies which attempt to correlate membrane physical probe data with membrane function in more complex systems.

The ideal organism for such model studies of membrane lipid properties would be wholly incapable of the synthesis of lipid precursors (e.g., fatty acids, squalene or choline) but would readily incorporate any such

precursors, or preformed lipids, into the membrane when they were exogenously supplied. A more realistic objective would be to generate a membrane system having a small number of lipid components whose structure could be varied almost at will within a minimal set of constraints imposed by the ability of various lipid species to support proper membrane function and cell growth. The obvious starting point for developing such a 'lipid-simplified' membrane system is an organism whose lipid metabolism is fairly simple, whose membrane(s) can be easily isolated and, if necessary, separated from one another, and whose lipid requirements stem mainly if not wholly from its need to generate simple membrane lipids. A good choice of organism in view of these criteria is the simple, cell wall-less prokaryote, Acholeplasma laidlawii B, which, along with E. coli, already provides the most readily manipulable plasma membrane system currently available (McElhaney and Tourtellotte, 1969; McElhaney, 1974; Saito and McElhaney, 1977).

The overall objective of the present work is to help to bridge the gap between the increasingly sophisticated studies of the physical properties of pure lipid-water systems which are now being reported by a number of laboratories, and a large number of interesting but highly qualitative and sometimes naïve studies of the physical properties of lipids in, and their effects on the functional properties of, the intact membranes of living cells. To this end, I have developed a method by which the fatty acyl composition of the membrane lipids of A. laidlawii B can be manipulated over a wide range and can be made essentially homogeneous in any of a number of fatty acids, and I have evaluated the function of a large variety of fatty acids in supporting cell growth when enriched to high levels in the membrane of this organism. This system for link-

ing physical and biological studies of membranes and lipids has been extended in the physicochemical direction by synthesizing and characterizing pure diacyl phosphatidylcholines whose acyl chains, or their homologues, can also be incorporated to high levels in the lipids of A. laidlawii B. Certain physical properties (the gel-to-liquid-crystalline phase transition parameters) of these lipids in aqueous dispersion have been evaluated, as have the transition temperatures and mixing properties of fatty acid-homogeneous lipids isolated from A. laidlawii B. The 'lipid-simplified' membrane system has been extended in the biological direction by studying the effect of membrane fatty acid composition on cell growth in a detailed and systematic manner and by investigating the effects of lipid physical properties on the activity of a key membrane enzyme, the (Mg^{2+},Na^{+})-ATPase, and the mechanism by which these lipid effects are exerted on the enzyme. The results obtained from these studies indicate that the 'lipid-simplified' A. laidlawii membrane system has a considerable potential for use in investigating and correlating the physical and the biological properties of a cell membrane in much greater detail than has previously been possible.

CHAPTER 2

MATERIALS AND METHODS

The methods described in this chapter are those which were used in the biological aspects of my thesis work. The methods used for the synthesis of fatty acids and phosphatidylcholines are described in Chapter 5.

Materials

Biochemicals - Straight-chain saturated and unsaturated fatty acids were obtained from Nu-Chek Prep. (Elysian, Minn.), while commercial cyclopropane or branched-chain fatty acids were products of Analabs (North Haven, Conn.). L-α-dipalmitoyl- and dioleoylphosphatidylcholine, L-α--glycerophosphorylcholine (cadmium chloride adduct), avidin, ATP (sodium and magnesium salts) and ADP (sodium salt) were products of Sigma (St. Louis, Mo.) and were of the highest purity available. Bovine serum albumin (Fraction V, fatty acid poor) was obtained from Miles Biochemicals (Elkhart, Ind.) and was further delipidated by the procedure of Chen (1967). Bacto-peptone, Bacto-heart infusion broth and Bacto-yeast extract were products of Difco (Detroit, Mich.). Cerulenin was the generous gift of Dr. Satoshi Ōmura (Kitasato Institute, Kyoto, Japan) who also furnished us with a small sample of CM-55.

Organic Chemicals - The various organic reagents used in the synthesis of fatty acids and phosphatidylcholines were obtained from Fisher Scientific (Edmonton, Alta.), Baker Chemical Co. (Phillipsburg, N.J.), Aldrich Chemical Company (Milwaukee, Wisc.) or K & K (Irvine, Calif.) and were all of reagent grade or higher purity. Acetylene gas was obtained from Union Carbide of Canada Ltd. (Oakville, Ont.). Cirrasol was a gift

from Imperial Chemical Industries, Ltd. (Manchester, U.K.). All organic solvents were of reagent grade and were routinely redistilled prior to use.

Inorganic Reagents - Anhydrous ammonia, hydrogen chloride and hydrogen gases were products of Union Carbide Canada Ltd. Common salts and buffers were obtained from Fisher Scientific Co., Baker Chemical Co. or Koch-Light Industries (Colnbrook, U.K.) and were all of reagent grade. Halogens and metals used in organic syntheses were purchased from Fisher Scientific Co. and were 'Fisher Certified' grade.

Chromatographic Adsorbents - Bio-Sil A (200-400 mesh) was obtained from Bio-Rad (Missisauga, Ont.), while silica gels G and H were obtained from E. Merck (Darmstadt, G.F.R.). These adsorbents were washed with chloroform and methanol, then dried to remove any organic contaminants prior to use. The gas-chromatographic columns used (10% diethylene glycol succinate on Anakron) were obtained from Analabs, while the gases used (ultra-high purity hydrogen, 'zero-gas' grade air and prepurified grade helium) were products of Union Carbide Canada Ltd.

Methods

Organism and Culture Conditions - Our isolate of Acholeplasma laidlawii B was originally obtained from Dr. G. ff. Edwards (Wellcome Research Laboratories, Beckenham, U.K.). The delipidated growth medium was prepared as described elsewhere (Silvius and McElhaney, 1978) and was supplemented with 0.4% (w/v) bovine serum albumin, 0.25% (w/v) glucose and 10^5 I.U./litre of penicillin G (all as sterile solutions) prior to inoculation of the medium. Fatty acids were added as solutions in a small quantity of ethanol (< 0.3% of the culture volume). Cultures were

grown statically at 36°C, and cell growth was monitored turbidometrically by absorbance at 450 nm (Maniloff, 1969). Cells were harvested by centrifugation at 13,000 x g for 15 min at 4°C in a Sorvall RC2-B centrifuge, using a GSA rotor.

Membrane Isolation - Our procedure was basically that of Pollack et al. (1965a). Cells grown to late log phase were harvested as just described, resuspended in one-half culture volume of 'β-buffer' (0.154 M NaCl, 0.05 M Tris, 20 mM β-mercaptoethanol, pH 7.4) and recentrifuged. The cell pellet was resuspended in one-fifth of the original culture volume of warm (35°) distilled water by vigorous trituration. After incubation at 35°C for 30 min, the suspension was centrifuged at 8,000 x g for 10 min to remove debris and whole cells, then the supernatant was centrifuged at 30,000 x g for 30 min using a Sorvall SS-34 rotor. The membrane pellet was washed once by recentrifugation from a volume of twenty-fold diluted β-buffer equal to the volume of distilled water used to lyse the cells. This procedure typically yielded 30 - 50 mg of membrane protein from 1 ℓ of cell culture.

Lipid Extraction - Our method is a modification of the method of Bligh and Dyer (1959). The sample of interest (whole cells or isolated membranes, containing up to 100 mg of protein) was suspended in 8 ml of 'α-buffer' (0.15 M NaCl, 0.02 M Tris, 0.01 M $MgSO_4$, pH 8.0), then 20 ml of methanol was added and the mixture was warmed to ∿50°C for a few minutes to denature the protein. Chloroform (30 ml), α-buffer (20 ml) and chloroform (40 ml) were then added sequentially, shaking the mixture well after each addition. The phases were then allowed to separate, the upper (aqueous methanol) phase was aspirated off, as was the interfacial material, and the lower (chloroform) phase was applied to a column of Bio-Sil A

(5 - 20 gm) packed in chloroform. The column was eluted with 5 column volumes of chloroform to yield the neutral lipids, then with 5 volumes of methanol to yield the polar lipids. In most experiments (e.g., glycerolipid fatty acid analysis) only the latter fraction was retained, but in some DTA experiments, both the neutral and polar lipids were collected together. Evaporation of the organic solvents in vacuo yielded the purified lipids, which were redissolved in the minimum quantity of benzene or chloroform/methanol (2:1, v/v).

Fractionation of Polar Lipids - A. laidlawii membrane lipids can be fractionated into neutral lipid, glycolipid and phospholipid components by eluting a column of Bio-Sil with 20 volumes each of chloroform, acetone and methanol, according to the procedure of Vorbeck and Marinetti (1965). However, we found that the resolution of glyco- from phospholipids by this method was not always complete, and we therefore carried out most of our polar lipid fractionations by thin-layer chromatography. Lipid samples of 0.5 - 20 mg were applied to 5 x 20 or 20 x 20 cm plates of silica gel G or H (0.5 mm thick) for synthetic phospholipids. The plates were developed with 70:25:5 (v/v/v) chloroform/methanol/water (for A. laidlawii lipids), or 50:50:2.5:2.5 (v/v/v/v) chloroform/methanol/water/conc. NH_4OH (for synthetic phospholipids). After the plates were developed, they were exposed briefly to I_2 vapor to visualize the lipid spots, which were then scraped off with a razor blade. The gel scraped from the plates was packed into a small chromatography column and eluted with 10 ml of 9:1 (v/v) methanol/chloroform to yield the pure lipid upon evaporation of the solvent. If recovery of the lipids from the plates was not required, the plates were sprayed with a 3% solution of chromium trioxide in 60% (w/w) aqueous sulfuric acid, then charred on a hot plate

to reveal the lipids as brown-to-black spots.

Lipid Fatty Acid Analysis - A lipid sample (up to 2 mg) was heated to 70° with 1 ml of 5% methanolic H_2SO_4 for 1 hr (for free fatty acids or lipids containing cyclopropane acyl species) or 2 hr (for other complex lipids). The cooled reaction mixture was mixed with 2 ml of distilled water and twice extracted with 1 ml hexane, which was dried (Na_2SO_4) and evaporated under N_2 to yield the fatty acid methyl esters. These were analyzed by gas chromatography on columns of 10% diethylene glycol succinate on Anakron in a Hewlett-Packard 5700 A gas chromatograph. The signal from the flame ionization detector (which is proportional to the mass of the organic components in the effluent) was fed into a Hewlett-Packard 3370 B integrator and displayed on a Hewlett-Packard 7128 A strip-chart recorder.

Gel Electrophoresis of Membrane Proteins - Membrane samples were dissolved in a buffer containing 1% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, and 50 mM phosphate (pH 7.0), then heated at 45°C for 60 min. Roughly 25 µg of protein was applied to a 6 x 20 x 0.3 cm slab gel of 8% polyacrylamide equilibrated with a buffer containing 6 M urea, 0.15% SDS and 30 mM phosphate pH 7.0. The gels were electrophoresed using bromophenol blue dye as a tracking dye, then stained with Coomassie blue and destained, as described by Weber and Osborne (1969). Gels were scanned at 596 nm on a Gilford model 250 spectrophotometer and the staining intensity (absorbance) was continuously recorded as a function of position in the gel.

Differential Thermal Analysis (DTA) of Lipid Samples - Samples of 5 - 15 mg of pure lipids were dissolved in 0.5 ml of warm benzene, which was rapidly frozen and lyophilized to give the lipid in a powdery or waxy

form. This was packed into capillary tubes, mixed with 50% (w/w) distilled water or, in a few cases, 70% (w/w) of 30% aqueous ethylene glycol, and hydrated by repeated warming and centrifugation of the sample from one end of the (sealed) capillary to the other (Ladbrooke et al., 1968). After a homogeneous opaque dispersion was obtained, the tube was broken in two and the half of the tube containing the sample was placed in the sample chamber of a DTA cell for a DuPont 900 Thermal Analyzer, using glass beads in the reference chamber. Samples were heated or cooled at rates from 2° - 20°C/min (depending on the nature of the experiment); transition temperatures were normally determined at scan rates of 3°/min (for synthetic phospholipids) or 5°/min (for A. laidlawii lipids).

Assay of the A. laidlawii Membrane ATPase Activity - The 'standard' procedure was as follows: isolated membranes (0.05 - 2.0 mg of protein) were incubated with 1 mM ATP, 50 mM Tris, 50 mM NaCl and 15 mM $MgSO_4$ at pH 7.6 in a total volume of 4 ml or 10 ml. At fixed time intervals, 0.5 or 1 ml aliquots of the digest were withdrawn and mixed with 50 or 100 µl, respectively, of 5% aqueous SDS, which completely quenched the ATPase activity. The samples withdrawn were assayed for inorganic phosphate by reaction with Cirrasol and acid ammonium molybdate as described by Atkinson et al. (1973).

Protein Assay - Samples of membrane containing roughly 5 - 50 µg of protein were made up to 1 ml with distilled water after addition of 50 µl of 0.4% aqueous Triton X-100. The samples were then assayed for protein by a modification of the procedure of Lowry et al. (1951) as described by Hartree (1972). Crystalline bovine serum albumin was used to prepare standard protein solutions.

CHAPTER 3

TOTAL REPLACEMENT OF THE NORMAL LIPID ACYL GROUPS OF A. laidlawii B BY EXOGENOUS SPECIES, AND EFFECTS ON CELL GROWTH

Background

Most free-living organisms can synthesize long-chain fatty acids de novo from short-chain alkanoic acids and acetate (Wakil, 1970; Volpe and Vagelos, 1976). This capability allows the organism to generate a 'background' of endogenously produced fatty acids which compete with an exogenously supplied fatty acid for incorporation into membrane lipids, making it virtually impossible to entirely replace the normal fatty acid species of the organism's membrane lipids with an arbitrary exogenous species in vivo. As it is highly desirable to manipulate the membrane lipid fatty acid composition as widely as possible in order to widely vary membrane lipid properties (and to observe the resulting effects on membrane function) in vivo, membrane workers have used two major approaches to reduce or abolish the capacity of various organisms for de novo fatty acid synthesis in vivo. The first approach is a genetic one, using organisms with mutations in one or more of the genes controlling fatty acid metabolism, and has been used with most success to construct strains of E. coli which are deficient in any or all of the pathways for fatty acid oxidation, de novo synthesis of fatty acids or synthesis of unsaturated fatty acids (Silbert, 1975). The second approach is a chemical one, using specific inhibitors instead of genetic lesions to produce the same end results. Because specific and nontoxic inhibitors of fatty acid synthesis have not been generally available, the chemical approach has been used less commonly than the genetic approach with prokaryotes, on which

most lipid-manipulation studies have focused to date. Nonetheless, the chemical approach has considerable potential, especially in systems which are difficult to manipulate by genetic means (e.g., in the Mycoplasmas), and the search for specific inhibitors of fatty acid synthesis *in vivo* clearly merits attention from membrane and lipid researchers.

To date, two compounds, cerulenin and avidin, have generally proven useful as inhibitors of fatty acid synthesis in a variety of systems, including both microorganisms and tissue culture cells. Cerulenin, a fungal antibiotic which inhibits the 'condensing enzyme' of the *de novo* biosynthetic pathway (Figure 1) (Ōmura, 1976), has been used most successfully *in vivo* in prokaryotes such as *A. laidlawii* (oral strain)(Rottem and Barile, 1976) and B strain (Saito *et al.*, 1977a, 1978)), *Staphylococcus aureus* (Altenbern, 1977) and *E. coli* (Goldberg *et al.*, 1973; Buttke and Ingram, 1978). However, cerulenin is costly and chemically labile, and it does not wholly abolish fatty acid synthesis at subtoxic levels in any organism yet tested. Therefore, its usefulness in lipid manipulation is somewhat limited. Avidin is a small protein which binds with very high affinity to biotin, an essential cofactor for the acetyl-CoA carboxylase reaction of the *de novo* fatty acid - biosynthetic pathway, as illustrated in Fig. 1 (Volpe and Vagelos, 1976). If an organism required biotin only for this reaction (or at least for no other reaction vital to the normal metabolism of the cell) and was auxotrophic for this coenzyme as well, then avidin, by sequestering biotin in a nonutilizable form, would strongly and specifically inhibit fatty acid synthesis by that organism. While many prokaryotes are biotin prototrophs, mammals are not, and avidin has been used with considerable success to inhibit fatty acid synthesis in mammalian tissue culture cells (Hatten *et al.*, 1977; Horwitz

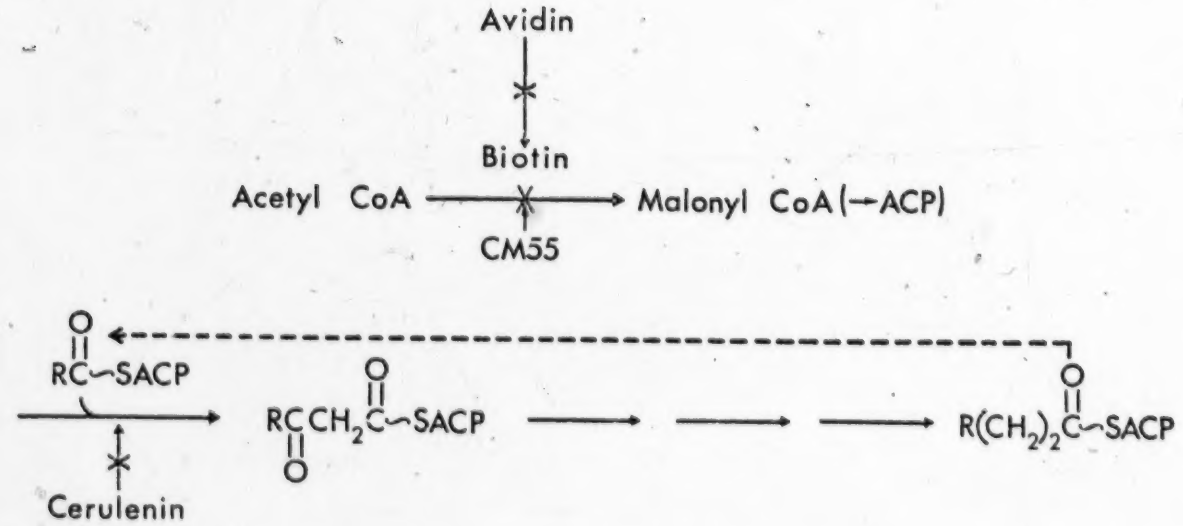


Figure 1. Schematic representation of the presumed de novo fatty acid biosynthetic pathway in A. laidlawii B, indicating the probable sites of inhibition by cerulenin, CM-55 and avidin. The abbreviations 'CoA' and 'ACP' stand for coenzyme A and acyl carrier protein, respectively.

et al., 1978). A few other antilipogenic compounds have been found to inhibit fatty acid biosynthesis in certain organisms. Ohno et al. (1974) have reported that N,N-dimethyl-4-oxo-2-trans-dodecenamide (CM-55) inhibits fatty acid production in yeast, probably by inhibiting acetyl-CoA carboxylase, although it also inhibits amino acid transport at useful dosages. α-Chlorophenoxyisobutyric acid (CPIB) has been reported to inhibit fatty acid and polyprenol synthesis in Tetrahymena pyriformis (Nozawa, 1973). These last two compounds have not been used as widely as have cerulenin or avidin, however, and neither has been used to modify membrane lipid composition for studies of the lipid role in membrane function.

As outlined in the Introduction, my goal in this study was to completely abolish the synthesis of fatty acids by A. laidlawii B in vivo, so that the membrane lipid fatty acid composition could be modified over a range comparable to that attainable with pure-lipid or reconstituted lipid-protein systems. Since mycoplasmas in general are quite refractory to genetic manipulation, in part because they form polykaryons (Smith, 1971), I chose to use chemical treatments to achieve this goal. To this end, I tested the effects of a number of substances on fatty acid synthesis by A. laidlawii B. When avidin was found to be an excellent inhibitor of fatty acid synthesis and chain elongation in this organism, I made use of the opportunity thus afforded to manipulate the membrane lipid acyl chain composition at will to investigate the effect of the acyl chain composition on cell growth. This latter study is the most thorough and rigorous of its kind in any system to date and sheds new light on the relationship of membrane lipid structure to membrane and cellular function.

Experimental Results and Implications

Chemical Inhibition of Fatty Acid Biosynthesis - Potential inhibitors were screened for usefulness *in vivo* by culturing cells on media supplemented with elaidic acid (0.10 mM) or palmitic plus oleic acid (0.05/0.05 mM) with various levels of the inhibitor, comparing the cell growth under these conditions with that of cultures grown on media with the inhibitor but without fatty acid. A useful inhibitor would be expected to decrease cell growth in the absence of exogenous fatty acids (reflecting a lack of fatty acids required for membrane lipid synthesis) but would have no effect on cell growth in the presence of fatty acids suitable for the synthesis of membrane lipid species compatible with membrane function. My choice of elaidate ($18:1t\Delta^9$) and palmitate (16:0)/oleate ($18:1c\Delta^9$) as 'suitable' exogenous fatty acids was initially no more than an educated guess based on previous lipid-manipulation studies with this organism (Saito and McElhaney, 1977); happily, these choices were shown to be correct by my subsequent studies (*vide infra*). Using the screening method just outlined, I rejected a number of halogenated analogues of short-chain fatty acids (including iodoacetate, 3-chloro-, 3-bromo- and 3-iodopropionate and 3-bromobutyrate) as well as CPIB and 4-pentenoic acid. Three compounds, cerulenin, CM-55 and avidin, were selected for further study.

To quantify the inhibition of fatty acid synthesis by a given compound, I resorted to direct analysis of the fatty acid composition of the membrane lipids of cells cultured with a *de novo* biosynthetic primer, an exogenous long-chain fatty acid and varying levels of the compound of interest. The exogenous long-chain acid was always elaidate ($18:1t\Delta^9$), isopalmitate (16:0i) or palmitelaidate ($16:1t\Delta^9$), all of which support normal cell growth when they constitute essentially all of the membrane

lipid acyl chains, as we shall see below. The primer was normally propionate (3:0), isobutyrate (4:0i) or isovalerate (5:0i), all of which are well-utilized by the de novo biosynthetic system of A. laidlawii B (Saito et al., 1977a) to give end-products which are easily identifiable by GLC. As the concentration of inhibitor increases, the intracellular steady-state ratio of these de novo end-products to the exogenous fatty acid will fall, and the level of the exogenous species in the total membrane lipid acyl groups will rise at the expense of the endogenous species. This effect is shown for CM-55 at three temperatures in Figure 2. Note that only at 21°C (where cell yields are only about one-third of maximal) does CM-55 reduce the de novo biosynthetic output to negligible levels at subtoxic concentrations (< 5 µg/ml). In Figure 3, the results of a similar experiment using avidin at 35°C are shown. It is clear from a comparison of the results with CM-55 and with avidin at 35°C that the latter is the inhibitor of choice for A. laidlawii B (no nonspecific toxicity was detected up to 4 mg/l, which is well above the effective dosage). Cerulenin is less potent than either CM-55 or avidin in inhibiting de novo fatty acid biosynthesis (Rottem and Barile, 1976; Saito et al., 1977a). The effectiveness of avidin in suppressing endogenous fatty acid production and thereby facilitating manipulation of the composition of membrane lipid fatty acyl chains in A. laidlawii B is illustrated by the data of Table 1, where the fatty acid compositions of several cultures grown with various fatty acids plus avidin are given. It is evident that the problem of an endogenously synthesized fatty acid 'background' which dilutes out the exogenous species in the membrane lipids can be essentially abolished by the use of avidin.

In addition to synthesizing fatty acids de novo, A. laidlawii B

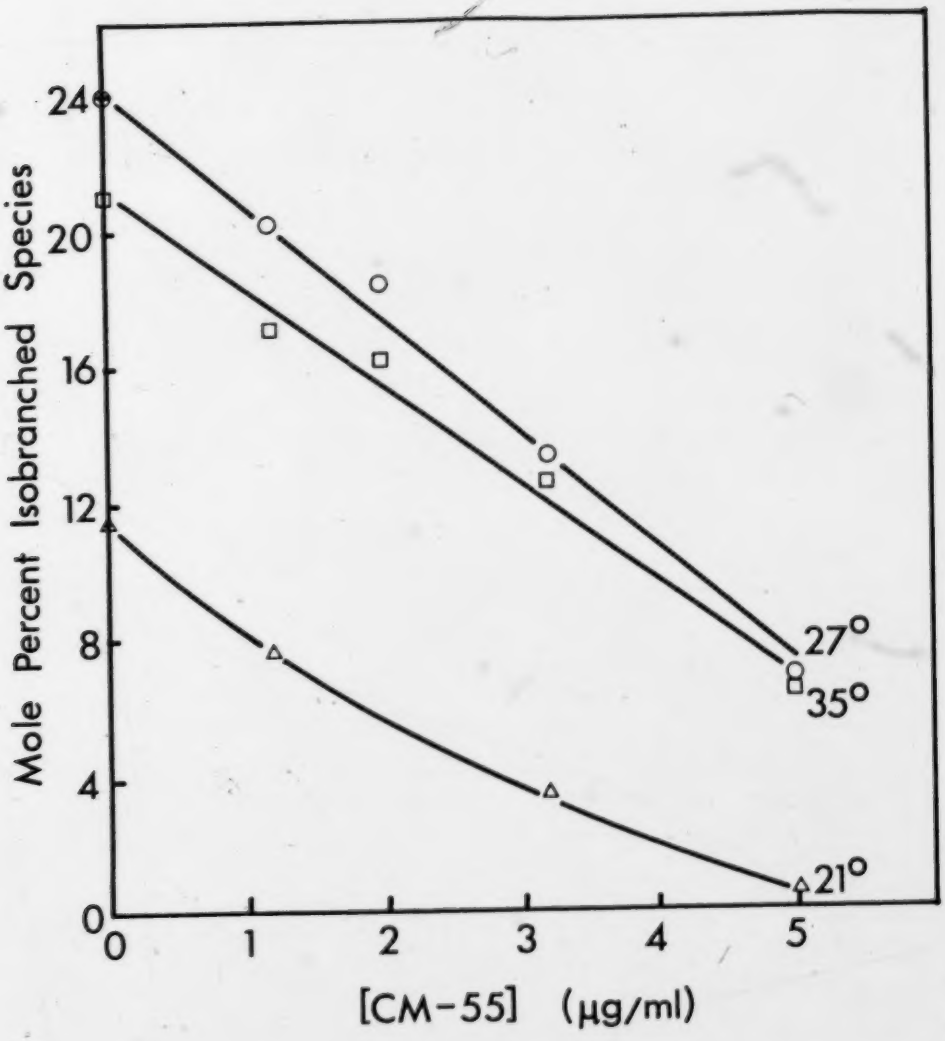


Figure 2. Inhibition by CM-55 of incorporation of a primer of de novo fatty acid synthesis, isovaleric acid, into the fatty acyl chains of membrane lipids in cells grown with 0.10 mM palmitelaidic acid ($16:1t\Delta^9$) plus 1 mM isovaleric acid (5:0i) at three temperatures (shown on graph). Results are expressed as the molar percentage of odd-chain isobranched fatty acyl chains (those derived from isovaleric acid) in the membrane lipids of cells grown with various levels of CM-55, determined as described in the text.

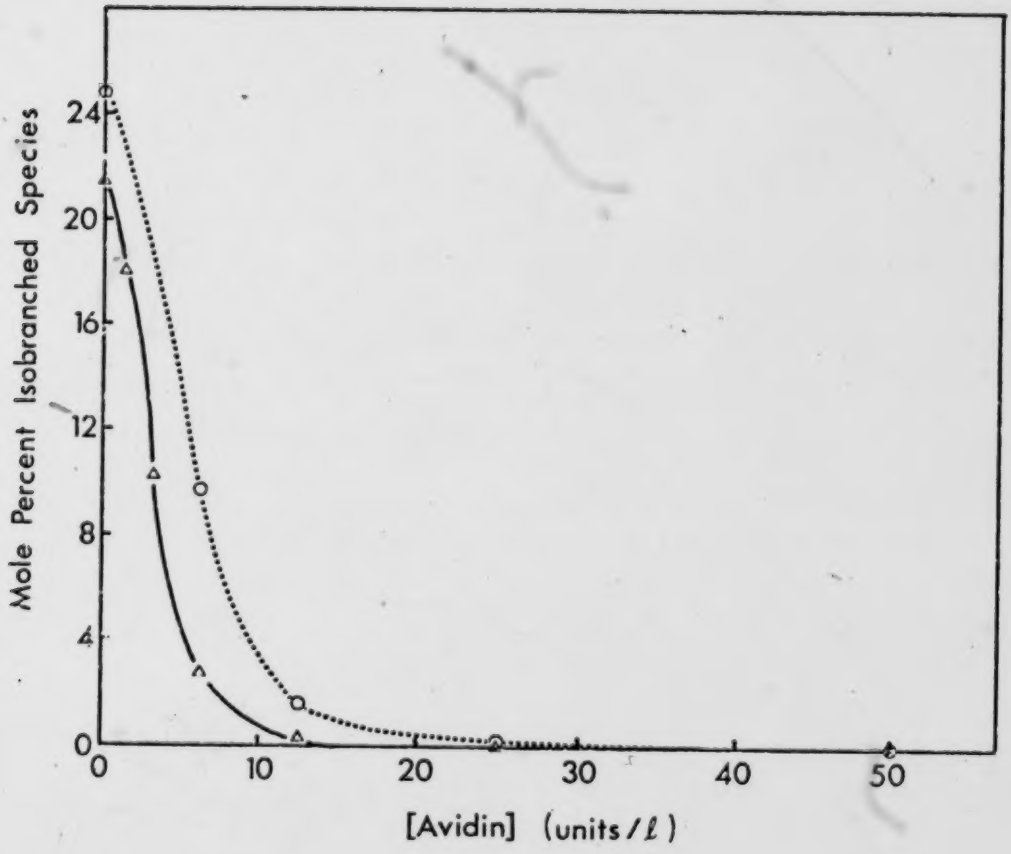


Figure 3. Effect of avidin dosage (1 mg avidin = 25 units) on the de novo biosynthesis of long-chain fatty acids from isovaleric acid (5:0i) in cells grown with 1.0 mM isovaleric acid plus 0.1 mM palmitelaidic acid (16:1tΔ^9). Details of the experiments are essentially as described for Figure 2.

Table 1

Fatty Acid Composition of Glycerolipids from A. laidlawii B Grown with Various Fatty Acids Plus 2 mg/l Avidin at 37°C

Mole % in Lipids of:	Exogenous Species				
	14:0i[a]	16:0i	17:0ai	16:1tΔ9	16:0/18:1cΔ9
12:0	-[b]	0.2	0.2	0.2	0.2
14:0i	95.4	-	-	-	-
14:0	0.1	0.2	0.2	0.2	0.3
16:0i	1.3	95.5	-	-	-
16:0	0.9	1.5	0.9	0.8	50.4
16:1	-	-	-	97.0	-
17:0ai	-	-	96.1	-	-
18:0	0.9	1.3	1.1	0.7	0.1
18:1	0.8	0.7	0.8	0.6	48.7
18:2	0.6	0.6	0.8	0.5	0.3

[a] Fatty acids were added to the culture medium at a concentration of 0.12 mM (for single species) or 0.06 mM each (when two fatty acids were added).

[b] '-' denotes undetectable levels (< 0.02% of total fatty acids).

can also add one or a few two-carbon units to exogenous medium-chain (eight to fifteen-carbon) fatty acids, thereby increasing the suitability of the exogenous acids for utilization in complex lipid biosynthesis (Saito et al., 1978). While all three of the inhibitors described above inhibit fatty acid chain elongation to some extent, avidin is clearly superior to the other two; thus at levels of each inhibitor which give optimal inhibition of de novo fatty acid synthesis without loss of cell viability, the elongation of myristoleic acid ($14:1c\Delta^9$) is inhibited by 48%, 45% and ~95% by cerulenin, CM-55 and avidin, respectively. In Table 2 are given the fatty acid compositions of cells grown with or without avidin in the presence of two fatty acids which are normally substrates for the fatty acid elongation pathway, illustrating the potency of avidin in suppressing exogenous fatty acid chain elongation. In general, fatty acid elongation is somewhat more resistant to chemical inhibition than is de novo biosynthesis. This observation does not necessarily contradict our previous suggestion that the 'elongation pathway' is simply another aspect of the de novo pathway, for it is quite possible that the latter pathway, which requires five to seven repetitions of the (two-carbon) chain-elongation cycle (Figure 1) to produce a molecule of product, is more sensitive to a partial inhibition of this cycle (induced by reducing the levels of a key substrate, malonyl-ACP) than is the elongation pathway, which requires only one or two passages through the cycle to yield its end-products. Using avidin, it is possible to essentially abolish chain elongation as well as de novo biosynthesis in almost all cases, while the few exceptions are invariably found to be cultures which are supplemented with fatty acids giving very poor growth and are thus unlikely to be of practical consequence.

Table 2

Effect of Avidin (2 mg/ml) on Fatty Acid composition of Lipids from Cells Cultured with Two Elongation Substrates

Mole % in Lipids of:	Culture Supplements			
	14:0i[a]	14:0i/Avidin	15:0ai	15:0ai/Avidin
12:0	0.9	-[b]	0.5	-
13:0	0.2	-	0.1	-
14:0i	55.3	95.6	-	-
14:0	6.6	0.1	6.2	0.3
15:0ai	-	-	59.0	94.9
15:0	0.6	-	-	-
16:0i	18.5	1.3	-	-
16:0	10.7	0.9	12.5	1.0
17:0ai	-	-	17.4	0.8
17:0	0.2	-	-	-
18:0i	0.2	-	-	-
18:0	3.8	0.9	1.0	1.4
18:1	2.1	0.8	2.6	0.9
18:2	0.9	0.6	0.6	0.7

[a] Fatty acids were added to the culture medium at a concentration of 0.12 mM.

[b] '-' denotes undetectable levels (< 0.02% of total fatty acids) of the species in question.

From these experiments, we conclude that avidin is a suitable agent for totally inhibiting fatty acid chain generation or elongation by A. laidlawii B. This organism has no ability to modify acyl chain lengths by degradation either, as it lacks the β-oxidation pathway (Lynn, 1960). Therefore, when grown with avidin plus an exogenous acid, the cells must either synthesize membrane lipids whose acyl chains are essentially pure unmodified exogenous acid (allowing for a slight incorporation of contaminating fatty acids from the growth medium, which can never be entirely delipidated) or die from a failure to synthesize adequate amounts of membrane lipids (and hence of membrane) to keep pace with other aspects of cell growth. The production of 'fatty acid-homogeneous' membranes using avidin and a single exogenous fatty acid is thus feasible so long as the exogenous species generates lipids which support adequate membrane function for proper cell growth (we shall investigate the criteria for a 'suitable' exogenous acid in the next section). While CM-55 is less useful than avidin in abolishing fatty acid synthesis in A. laidlawii B, it may prove to be a valuable alternative to cerulenin for manipulating lipid fatty acyl composition in organisms which are biotin prototrophs and would therefore not respond to avidin treatment.

Growth of A. laidlawii B on Media Containing Avidin and One Fatty Acid - As noted above, if avidin is used to inhibit fatty acid metabolism, the only remaining factor limiting the possible extent of fatty acid manipulation in A. laidlawii B membrane lipids is the ability of various exogenous fatty acids to support cell growth. To evaluate the stringency of the constraints that this factor imposes on lipid manipulation in this system, I cultured cells on media containing avidin and any one of a large number of fatty acids and determined the cell growth rate and

yield for each culture. I then examined my results in the light of several known metabolic and physical properties of the fatty acids tested in an attempt to systematically explain the growth-supporting abilities of the various fatty acids in terms of a single key property or set of properties.

As a large number of samples were to be tested for cell growth, a simple yet reliable measure of cell growth was required. While measurements of particle counts or the amount of DNA in the sample would be feasible for small numbers of cultures, properly controlled measurements of these quantities were not convenient for the large numbers of samples I wished to analyze. I turned instead to measuring culture turbidity (at 450 nm) as an index of cell growth. Maniloff (1969) has reported that turbidity accurately reflects the viable counts in a culture in the log phase (but not in the stationary phase) of growth, and Rodwell (1967) has reported that culture DNA levels and turbidity are well correlated during growth. Furthermore, we have found that the maximum turbidity attained by cultures grown with various fatty acids but without avidin is independent of the nature of the exogenous fatty acid (except in the case of a few long-chain species, such as stearate (18:0), which are lytic), although the cellular morphology (seen by phase-contrast microscopy) of different cultures varies greatly. Therefore, culture turbidity seems to be an adequate and convenient index of cell growth, and the maximum turbidity of the cell yield, in this system.

The fatty acids tested for growth-supporting activity in avidin-treated cultures of A. laidlawii B can be loosely grouped into four classes, as has been done in Table 3. The good/fair/poor/very poor classifications used in this table may seem somewhat artificial, for the cell

Table 3

Classification of a Variety of Fatty Acids with Respect to Ability to Support Growth of A. laidlawii B When Added to Cultures Along with 2 mg/ml Avidin

Class I[a]	Class II	Class III	Class IV
15:0	14:0	13:0	12:0
14:0i	18:0i	20:0i	16:0
15:0i	dl 19:0ai	dl 13:0ai	17:0
16:0i	ℓ 19:0ai	ℓ 13:0ai	18:0
17:0i	18:1cΔ^{3}	dl 21:0ai	12:0i
dl 14:0ai	18:1cΔ^{5}	18:1cΔ^{6}	14:1tΔ^{9}
dl 15:0ai	18:1cΔ^{12}	18:1cΔ^{17}	18:2t,tΔ^{9,12}
dl 16:0ai	18:1cΔ^{13}	22:1cΔ^{13}	14:1cΔ^{9}
dl 17:0ai	18:1c ^{16}		16:1cΔ^{9}
dl 18:0ai			17:1cΔ^{10}
ℓ 15:0ai			18:1cΔ^{7}
ℓ 17:0ai			18:1cΔ^{8}
16:1tΔ^{9}			18:1cΔ^{9}
18:1tΔ^{6}			18:1cΔ^{10}
18:1tΔ^{9}			20:1cΔ^{11}
18:1tΔ^{11}			18:2c,cΔ^{9,12}
18:1cΔ^{4}			18:3c,c,cΔ^{9,12,15}
18:1cΔ^{11}			20:4c,c,c,cΔ^{5,8,11,14}
18:1cΔ^{15}			17:0cp,cΔ^{9}
19:cp,tΔ^{9}			17:0cp,tΔ^{9}
19:cp,tΔ^{11}			19:cp,cΔ^{9}
			19:cp,cΔ^{11}

[a]Class I fatty acids give > 70% of normal growth, Class II, 30 - 70% of normal growth, and Classes III and IV, 10 - 30% and < 10% of normal growth, respectively.

yield (maximal turbidity) is, after all, a continuous variable. However, the divisions thus achieved correlate with certain other differences in the properties of cultures grown with fatty acids of various classes. For example, Class I fatty acids give 'normal' cell morphologies (chains of roughly spherical units), while cultures grown on Class III species show many ghosts even before the culture turbidity has leveled off. The most experimentally useful fatty acids for producing 'fatty-acid-homogeneous' membranes are the fatty acids of Classes I and II, for these species support cell yields adequate to allow the researcher to obtain tens or even hundreds of milligrams (dry weight) of membranes from at most several litres of culture medium. Interestingly, while cell growth yields depend strongly on the nature of the exogenous fatty acid supplement, the growth rates do not if growth occurs at all.

What correlations can we make between the growth-supporting activities of a given fatty acid and its other properties, e.g., its physical properties or its metabolic utilization by A. laidlawii B in the absence of avidin? We cannot attribute the results of Table 3 to fatty acid toxicity, as of the species shown in the table, only stearate (18:0) and arachidonate (20:4) reduced yields below those of fatty acid-unsupplemented controls in cultures grown without avidin. Neither can we explain these results in terms of the varying abilities of the fatty acids to be utilized by the membrane lipid-synthesizing machinery of A. laidlawii B. For example, oleate ($18:1c\Delta^9$) and elaidate ($18:1t\Delta^9$) are incorporated to very nearly the same extent into membrane lipids in competition with a fixed level of palmitic acid (16:0) and have very similar positional specificity ratios for acylation of the 1- vs. the 2-position of the glyceryl moiety of membrane polar lipids (Saito et al., 1977b). From

these observations, one would conclude that oleate and elaidate would be utilized by the cells with roughly equal efficiency for membrane lipid synthesis and would thus support equally good growth under the above hypothesis. However, elaidate is a good growth substrate, oleate a poor one. A number of comparisons of this type have been made, and a significant fraction of these are in strong disagreement with the 'utilization efficiency' hypothesis.

To a lipid worker, particularly one interested in lipid physical properties, the data of Table 3 suggest an alternative hypothesis. Note that all of the Class I and Class II fatty acids have at least fourteen carbons. Artificial bilayers composed of dimyristoyl (14:0) phosphatidylcholine (PC) are proper semipermeable membranes, while dilauroyl (12:0) PC bilayers are quite leaky (Hauser and Barratt, 1973; Mandersloot et al., 1975). The fourteen-carbon lower limit to the size of Class I and II fatty acids could, therefore, arise from a need to create a sealed, non-leaky bilayer from the membrane lipids synthesized from them. Consistent with this proposal is the observation that all polyunsaturated species tested are found to be Class IV (very poor) growth substrates, for polyunsaturated phospholipid bilayers are considerably more leaky than their saturated or monounsaturated counterparts (De Gier et al., 1968). Therefore, an elementary physical property of the fatty acid, namely the leakiness, or perhaps more generally the 'fluidity', that it confers upon bilayers of lipids derived from it, can be of some use in predicting its growth-supporting abilities in our system. A second important fatty acid physical property, related to the first, is the gel-to-liquid-crystalline transition temperature (T_c) of the membrane lipids derived from a given fatty acid. This property affects cell growth because cells cannot grow

well when their lipids are largely in the gel state and cannot grow at all when their lipids are all in this state (McElhaney, 1974a; Jackson and Cronan, 1978). If we consider this property of fatty acids, we can understand why elaidate ($18:1t\Delta^9$) supports cell growth while stearate (18:0) does not, for dielaidoyl PC has a T_c of 12° while distearoyl PC has a T_c of 58°. As we shall see in a subsequent chapter, the T_c's of pure diacyl phosphatidylcholines (PC's) are fairly well correlated with those of A. laidlawii lipids homogeneous in the same acyl group, a fact which justifies our extrapolation from PC's to membranes in predicting T_c values. On the basis of lipid T_c values, we can explain the fact that in the several homologous series of fatty acids tested, the growth-supporting activity invariably falls off sharply as the chain length exceeds a certain value, for the lipid T_c then substantially exceeds the growth temperature. The interested reader may wish to utilize the phosphatidylcholine T_c values reported in Chapter 5 to make (and test) predictions regarding the abilities of other fatty acids listed in Table 3 to support cell growth at 35° on the basis of the low-gel-lipid requirement for proper membrane function.

Perhaps my most systematic study of the relationship of fatty acid structure and physical properties to cell growth-supporting ability in the presence of avidin involves the isomeric cis-octadecenoic (18:1c) acids. In Figure 4, the maximal growth yields of A. laidlawii B cultured with avidin plus each cis-octadecenoic acid positional isomer are shown, along with the transition temperature (T_c) of the phosphatidylcholine derived from each isomer (Barton and Gunstone, 1975). Saito and McElhaney (1978) have found that the P_2/P_1 ratio, an index of the relative preference of the exogenous species for selective acylation of the glycerol

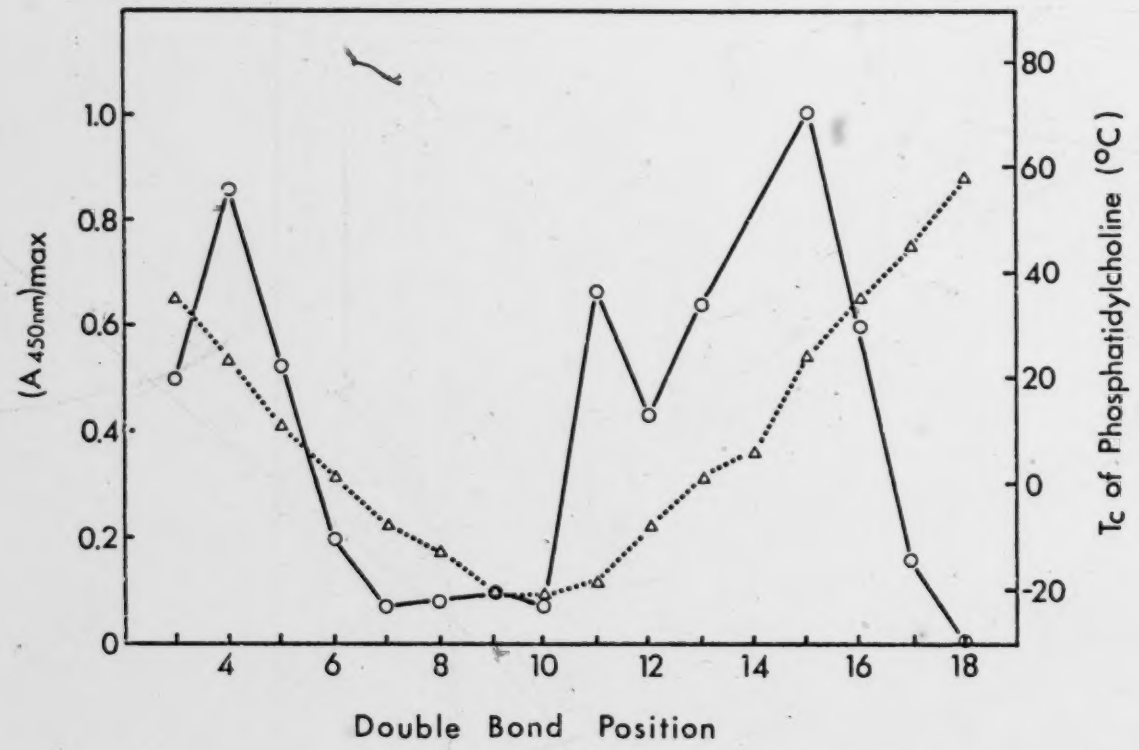


Figure 4. A. laidlawii B growth-supporting activity, and phase transition temperatures of derived phosphatidylcholines, of various isomer cis-octadecenoic acids. Solid line: relationship of maximal culture turbidity to double bond position when cells are grown with 2 mg/l avidin plus single cis-octadecenoic acids (0.10 mM). Broken line: gel-to-liquid-crystalline phase transition temperatures (T_c) of dioctadecenoyl phosphatidylcholines (Barton and Gunstone, 1975) vs. the position of the double bond. Details of the measurements are given in the text.

2-position in A. laidlawii B lipids, tends to increase as the double bond position approaches the carboxyl group in the series of cis-octadecenoic acids. The dependence of cell growth yields on double bond position in the experiments summarized in Figure 4 clearly follows the pattern of phosphatidylcholine T_c's more closely than it follows the variation of the P_2/P_1 ratio with the position of unsaturation. Cell growth is best on cis-octadecenoic acids whose derived phosphatidylcholines have phase transition temperatures (T_c) between roughly 0°C and 35°C. Therefore, the physical properties of the fatty acids as components of membrane glycerolipids appear to be more important than the details of their metabolism by the cells in determining their growth-supporting ability. The extents of lipid incorporation of the different isomers in competition with an equimolar concentration of exogenous palmitate, which provide estimates of the overall rates of utilization of each species for lipid synthesis, do not show any significant correlation with the cell growth-supporting ability of the cis-octadecenoic acids, as was generally noted above. Interestingly, in some cases two different cis-octadecenoic acids giving similar phosphatidylcholine T_c's give rather different cell growth yields, and in general, species with their double bonds in the half of the acyl chain nearer the methyl end give better growth than those with their double bonds closer to the carboxyl end. This observation suggests that subtle structural factors may affect the ability of various fatty acyl chains to support proper membrane function in a manner that is not directly reflected in the overall 'fluidity' or the gel-to-liquid-crystalline transition temperature of the lipids.

The results of the experiments summarized in Table 3 are heartening, for they indicate that it is possible to obtain substantial yields of

cells or membranes whose lipids can be enriched to near-homogeneity in any of a variety of fatty acids by using avidin. Our observations also lead us to the working hypothesis that it is the physical properties that the membrane lipid-incorporated fatty acid confers upon the lipids that determine whether or not that fatty acid is a suitable growth substrate for A. laidlawii B in the presence of avidin. I shall provide further evidence to support this contention in this and the next chapter.

Growth of A. laidlawii B on Avidin Plus Pairs of Fatty Acids - The discussion above leads us to the conclusion that an exogenous fatty acid supports, or fails to support, proper growth of A. laidlawii B in avidin-treated cultures because it causes the cells to generate membrane diacyl glycerolipids which are or are not compatible with proper membrane function. If this is true, then if cells could generate mixed-acyl lipids which support proper membrane function from two fatty acids which do not serve to support cell growth separately, the two fatty acids in combination should support cell growth in the presence of avidin. The best combinations of two fatty acids for generating membrane lipids of 'acceptable' physical properties would probably balance the properties of the two acyl species to give mixed-acyl lipids of physical properties near those of lipids containing only Class I fatty acyl chains. Thus palmitate (16:0) and stearate (18:0) would not be a good combination, as they both tend to product lipids whose phase transition temperatures T_c lie above the growth temperature, while palmitate and oleate ($18:1c\Delta^9$) would be a good combination, as the combination of palmitoyl and oleoyl chains on a single lipid molecule will give it a T_c below the growth temperature but will avoid the deleterious membrane effects of 'hyperfluid' diunsaturated lipid species (Davis and Silbert, 1974; Baldassare et al., 1977). In

fact, equimolar palmitate and oleate, when added to cultures of A. laidlawii B treated with avidin, do support good cell growth (data not shown), while palmitate plus stearate, and oleate plus laurate (12:0), give no growth at all. These results support our hypothesis that the physical properties of the (exogenously derived) acyl chains of the membrane lipids determine the extent of stimulation of cell growth by exogenous fatty acids in the presence of avidin.

If two fatty acids, A and B, of different and complementary physical properties are added to avidin-treated cultures of A. laidlawii B, one would expect the cell growth response to vary with the ratio of A to B added. At low levels of B, the membrane lipids will be mostly AA species, with some AB (or BA) species present. At roughly equimolar levels of A and B, most of the membrane lipids will be AB (or BA), for if A and B are significantly different in physical properties, the cells' metabolic machinery will generate the mixed-acyl species in preference to AA or BB species (McElhaney and Tourtellotte, 1970; Saito et al., 1977b). At high levels of B, the major lipid species will be BB with some AB (or BA) and present. The cell growth at varying levels of A and B will reflect the ability of AA, AB (or BA) and BB lipids to support proper membrane function.

If the two fatty acids A and B are both poor growth substrates by themselves but can combine to generate mixed-acyl lipids which can support proper membrane functioning, cell growth will rise as B is added in increasing amounts to cell cultures containing A at levels such that the concentration of A plus B is constant, peaking at a roughly equimolar A/B ratio and declining as the relative amount of B rises still further and BB lipid species accumulate in the membrane. I have shown that this

predicted result is correct by experiments using a variety of combinations of 'fluidizing' and 'rigidifying' fatty acids. One set of experimental results is shown in Figure 5, where cells were grown on varying ratios of tridecanoate (13:0) and palmitate (16:0) at a total concentration of 0.12 mM. In general, when cells are cultured with large amounts of a 'fluidizing' fatty acid and small amounts of a 'rigidifying' one, the incorporation of the former species into the membrane lipids is nearly constant as the level of the latter in the medium is changed, while the incorporated levels of the two species vary with their concentration ratio in the growth medium when growth is not limited by the supply of 'rigidifier'. This observation is illustrated by the data of Figure 5; note that tridecanoate levels in the lipids plateau at high levels of tridecanoate (low palmitate) but vary steadily with the levels of tridecanoate in the medium when palmitate is not growth-limiting. I interpret this result as follows. When the amounts of AA or BB lipids in the membrane exceed limiting tolerable levels, the cells die, so that cells grown at low levels of A or B are growth-limited by that species, and cell growth ceases when the cells cannot take up enough of the limiting species to generate an acceptable level of mixed-acyl lipids and dilute out the BB or AA lipids. When sufficient A and B are present to generate acceptable levels of AB (or BA) lipids throughout the normal course of cell growth, the cells incorporate A and B into membrane lipids in proportion to their levels in the growth medium, so that the A/B ratio in the lipids closely matches their ratio in the medium.

Determinations of the fatty acid composition of membrane lipids in cells grown at different A/B ratios, and of the growth yield for cells whose lipids contain equal levels of A and B, enable us to evaluate the

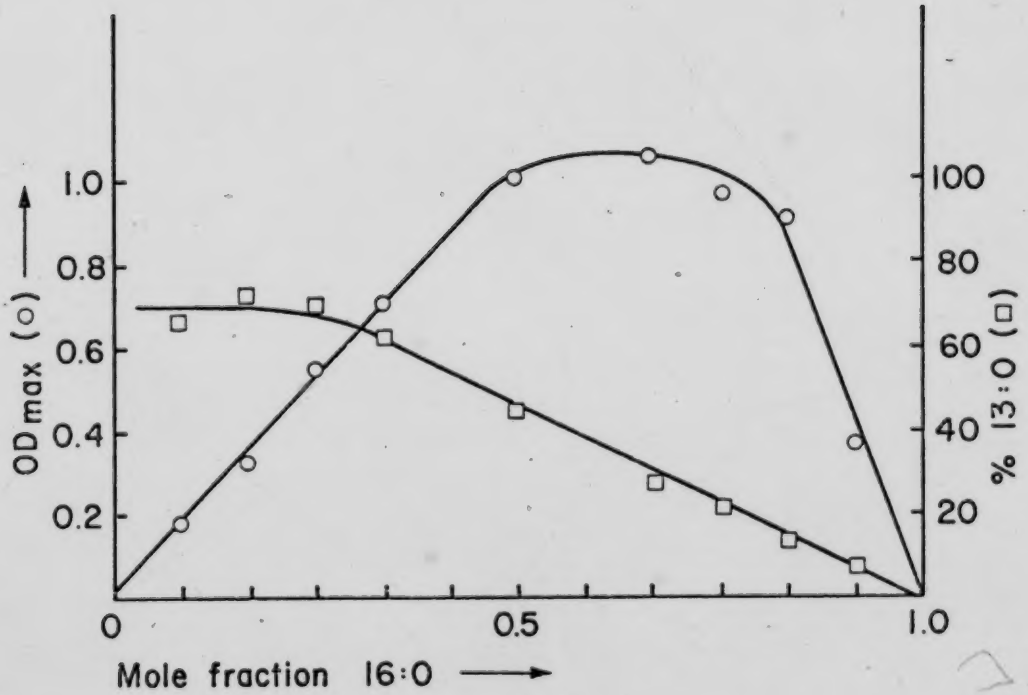


Figure 5. Growth yields, and tridecanoic acid (13:0) incorporation into membrane lipids, for A. laidlawii B grown with avidin (2 mg/l) plus palmitic (16:0) and tridecanoic acids in various concentration ratios and at a total concentration of 0.12 mM. Experimental details are given in the text.

abilities of AA, BB and AB (BA) lipids to support membrane function. From the limiting fatty acid compositions of cells grown at low levels of A or B, we can calculate the maximum tolerable levels of BB or AA lipids in the membrane using a calculation outlined in Appendix 2. From the extent of growth of cells containing equimolar A and B levels in the membrane lipids, we can calculate the suitability of AB (or BA) lipids to support membrane-linked processes essential for growth, for as noted above, AB (or BA) lipids will be the major membrane species synthesized under these conditions if A and B are substantially different in physical properties. The results of experiments designed to provide data for these analyses of membrane lipid 'functionalities' are described below.

My studies of the maximum tolerable levels of AA or BB lipids in the membrane focused on the case where the acyl species in excess is a 'fluidizing' species. Cells were cultured with palmitate (16:0), which served as a standard 'rigidifying' species, and varying levels of various 'fluidizers' at a constant total fatty acid concentration of 0.12 mM. As noted above, the maximal cell yield invariably became palmitate-limited at low levels of this acid, rising linearly with palmitate concentration; under these conditions, the levels of the 'fluidizer' in the membrane were very nearly constant. The limiting level of 'fluidizer' incorporation was different for various 'fluidizer' species but very similar when the 'rigidifier' was changed from palmitate to myristate, pentadecanoate or heptadecanoate (for example, the maximal incorporation of linoleate was 60%, 66% and 60% in the presence of myristate, pentadecanoate and palmitate, respectively). Therefore, the limiting levels of 'fluidizer' incorporation seem to be a characteristic property of the fluidizing species itself. Using the calculation outlined in Appendix 2, which takes

account of the positional specificity of incorporation of various acyl species into lipid molecules (McElhaney and Tourtellotte, 1970; Saito et al., 1977b), we can calculate the limiting (maximum tolerable) levels of lipids containing two 'fluidizer' chains from the limiting level of 'fluidizer' incorporation. In Table 4, these limiting values are given for various fatty acids, which are ranked according to the maximum level of lipids containing two such acyl chains which can be accommodated in the membrane without loss of cell viability. These results support the conclusions obtained from the growth studies using single fatty acids. Thus less dilauroyl (12:0) lipid than ditridecanoyl (13:0) lipid can be accommodated in the membrane, in agreement with the general tendency of the growth-supporting ability of a fatty acid to decline as its chain length decreases below fourteen carbons, which was noted in the last section. Likewise, less dilinoleoyl ($18:2cc\Delta^{9,12}$) lipid than dioleoyl ($18:1c\Delta^{9}$) lipid is tolerable in the membrane, in agreement with the tendency of increasing fatty acyl unsaturation to decrease cell growth-supporting ability. The results in Table 4 thus represent a refinement of the data of Table 3 regarding the classification of 'fluidizing' fatty acids in terms of their ability to support membrane function and cell growth.

Measurement of the maximal growth yield of cells whose membranes contain roughly equimolar amounts of fatty acids A and B, and hence contain mainly AB (or BA) lipids, allows us to evaluate the compatibility of various types of acyl groups when they are joined together in a lipid molecule. To this end, cells were cultured with various combinations of 'rigidifying' and 'fluidizing' fatty acids of many different structures, and the maximal cell yield was determined turbidometrically under conditions where the molar ratio of the two acyl species in the membrane lipids

Table 4

Maximum Tolerable Content of Various 'Fluidizing' Fatty Acids, and of Glycerolipids with Two Such Acyl Chains, in the Membrane of *A. laidlawii* B

Fatty Acid (X)	Maximum Lipid Incorporation of X (% Total Acyl Chains)	Maximum Levels of di-X Glycerolipids (% Total Glycerolipids)
18:1cΔ^{6}	82	65
18:1cΔ^{9}	79	59
14:1tΔ^{9}	77	56
16:1cΔ^{9}	76	55
17:1cΔ^{10}	76	52
20:1cΔ^{11}	76	52
13:0	73	49
22:1cΔ^{13}	64	28
12:0	59	25
19:cp,cΔ^{9}	60	24
18:2c,c$\Delta^{9,12}$	60	24
13:0ai	56	20
14:1cΔ^{9}	54	20
18:3c,c,c$\Delta^{9,12,15}$	54	15
12:0i	48	13

was near unity. The lipid fatty acid composition was determined for cultures harvested in the late log phase of growth, retaining a small part of the culture to complete the measurement of the growth curve into the stationary phase. The maximal growth yields (as percentages of control yields) for various combinations of fatty acids are given in Table 5. It can be seen that while straight-chain and isobranched fatty acids are compatible with all other fatty acyl structures tested here, anteisobranched, cyclopropane and unsaturated fatty acids do not combine with one another quite so readily to support good cell growth. Most notably, fatty acids with *cis*- double bonds near the centers of their acyl chains do not combine with other unsaturated or cyclopropane fatty acids to support good cell growth, even when the latter give relatively high transition temperatures for phospholipids derived from them (Barton and Gunstone, 1975). This finding may indicate that subtle structural factors other than the basic 'fluidizing' or 'rigidifying' character of a fatty acid may play a role in determining its cell growth-supporting abilities, as our data with the isomeric *cis*-octadecenoic acids (*vide supra*) also suggest.

One notable structural property of a fatty acid which could in principle affect membrane properties by means other than 'fluidity' changes is its hydrocarbon chain length, which could affect the membrane bilayer thickness and might thereby affect the disposition of membrane proteins and their biological activities. To test this possibility, I cultured cells with various combinations of fatty acids, one of each pair a 'rigidifier' and the other a 'fluidizer', which were chosen to create a series of membranes of varying average lipid hydrocarbon chain length. The results of these experiments are shown in Table 6; it can be seen that

Table 5

Growth of A. laidlawii B (As Percentage of Control) with Avidin Plus Various Pairs of Fatty Acids of Different Hydrocarbon Chain Structures

'Fluidizing Species'[a]	'Rigidifying' Species[a]						
	16:0	18:0i	19:0ai	18:1t-Δ^{11}	18:1c-Δ15	18:1c-Δ^{3c}	19:cp,t-Δ^{9c}
12:0	100[b]	80	89	90	94	93	94
13:0i	84	103	103	94	100	84	87
13:0ai	98	90	101	92	100	75	61
14:1tΔ^9	88	85	89	84	78	89	53
19:cp,cΔ^9	97	97	58	51	78	88	8
18:1cΔ^9	88	79	81	19	20	12	8
18:2c,c$\Delta^{9,12}$	88	75	20	12	<5	<5	<5

[a]Each fatty acid species was added to the culture medium at a concentration of 0.06 mM.

[b]Growth is quantified as the maximal turbidity at 450 nm attained by each culture, given as a percentage of the maximal turbidity attained by a culture grown without avidin or fatty acid supplementation. The final turbidities of replicate cultures generally agreed to within 10%, and in most cases, to within considerably closer limits.

[c]The studies of Barton and Gunstone (1975) indicate that cis-octadecenoic acids with the double bond near the carboxyl or methyl ends of the acyl chain are classifiable as membrane 'rigidifiers' on the basis of the rather high observed transition temperatures of their derived phosphatidylcholines.

Table 6

Growth of A. laidlawii B with Avidin Plus Various Pairs of Fatty Acids of Different Chain Lengths

Fatty Acids [a]	Incorporation Ratio [b]	Maximum Turbidity (% of Control) [c]
14:0/16:1cΔ^{9}	0.94	93
14:0/13:0i	1.62	84
15:0/17:1cΔ^{10}	0.84	82
16:0/18:1cΔ^{9}	1.01	85
17:0/20:1cΔ^{11}	0.76	102
18:0i/20:1cΔ^{11}	0.80	76
14:0/20:1cΔ^{11}	1.00	100
16:0/12:0i	0.98	100
16:0/14:1cΔ^{9}	0.72	76
18:0i/13:0ai	1.33	97

[a] Fatty acids were added to the culture medium at a concentration of 0.05 mM for each species. Pairs in the top group are considered to consist of species of like chain length, those in the bottom broup of species of unlike chain length.

[b] Given as the molar ratio of the first species to the second in the membrane lipids of late log phase cells.

[c] The reproducibility of turbidity measurements for replicate cultures is estimated to be ±5% in most cases, and ±10% in all cases.

good cell growth is possible when the mean extended hydrocarbon chain length of the lipids ranges from the equivalent of fourteen to seventeen all-*trans* methylene units. Therefore, it appears quite unlikely that the growth-supporting abilities of many (if any) of the fatty acids tested above depend primarily on their chain length *per se*. Experiments, also summarized in Table 6, which utilized pairs of fatty acids of unlike chain length as cell growth substrates showed that chain length mismatches of at least four to six all-*trans* methylene equivalents are tolerated by the cells without a substantial loss of viability, in agreement with the conclusion just presented. Therefore, the 'subtle structural determinant(s)' of fatty acid growth-supporting ability that were alluded to above do not seem to rest primarily on the acyl chain length.

Conclusions

The use of avidin with *A. laidlawii* B clearly offers a tremendously enhanced potential for the manipulation of cell membrane lipid fatty acyl composition and for the study of the relationship of the membrane fatty acyl composition to membrane lipid physical properties and biological functions. The rather flexible fatty acid requirements of avidin-treated *A. laidlawii* B allow the membrane lipid fatty acyl composition to be made essentially homogeneous in any of at least nineteen fatty acyl species by culturing cells with avidin plus a single fatty acid. Furthermore, a number of other fatty acids (e.g., oleic acid ($18:1c\Delta^9$)) can be enriched in the membrane lipids to the extent of 75% or more of total lipid acyl chains by also including small levels of a second fatty acid of complementary properties in the growth medium. It remains to be directly determined, of course, whether the changes thus induced in the

membrane lipid fatty acyl composition result in major changes in the physical properties (e.g., in the 'fluidity') of the membrane lipids, but I shall present evidence in the next chapter that at least one important property of the membrane lipids, their gel-to-liquid-crystalline transition temperature, is significantly altered by varying the nature of the Class I fatty acid with which the cells are cultured in the presence of avidin. Appreciable variations in membrane 'fluidity' at physiological temperatures are thus likely to occur in cells cultured with various Class I fatty acids plus avidin, but such changes cannot yet be considered as proven.

The ability of A. laidlawii B to grow when its membrane lipid fatty acyl chain composition varies within the very wide limits demonstrated here generally substantiates the previously stated suggestions (Cronan and Gelmann, 1975; Saito and McElhaney, 1977) that the lipid fatty acyl chains affect membrane function by modifying some bulk or average physical property of the membrane lipids, not by an intrinsic specificity of some membrane-related processes for lipids with acyl chains of a particular shape or (as I have shown here) hydrocarbon chain length. However, my results also give some indication that the specific configuration of lipid fatty acyl chains may affect their packing with other acyl chains to an extent sufficient to significantly modify membrane properties in a manner that cannot simply be attributed to overall 'fluidity' effects. Thus, for example, cis-15-octadecenoic acid, with an effective extended chain length and a phosphatidylcholine T_c very similar to those of palmitic acid (Barton and Gunstone, 1975), does not give good cell growth in combination with oleic (cis-9-octadecenoic) acid in the presence of avidin, while palmitic acid does. Observations of this type may reflect the

biological consequences of the recently demonstrated effects of specific moieties in lipid acyl chains (e.g., cis- double bonds or methyl branches) on the detailed nature of the transmembrane 'fluidity gradient' as determined in pure lipid bilayers by magnetic resonance and fluorescence methods (Seelig and Seelig, 1977; Seelig and Waespe-Šarčevič, 1978; Thulborn et al., 1978). If so, my results indicate that the concept of membrane 'fluidity' may require considerable refinement in order to understand how membrane lipid dynamic and orientational properties can affect the overall function of the membrane.

CHAPTER 4

COMPOSITIONAL AND PHYSICAL PROPERTIES OF ACHOLEPLASMA LAIDLAWII B MEMBRANES CONTAINING A SINGLE FATTY ACYL SPECIES

Background

The data of the preceding chapter indicate that by using avidin and exogenous fatty acids, it is possible to manipulate the membrane lipid fatty acid composition of A. laidlawii B more extensively than has been previously reported in any living system. The chief application of this ability to alter the lipid fatty acid composition will probably be the manipulation of the physical properties, such as the 'fluidity', the gel-to-liquid-crystalline transition temperature, or the permeability, of the membrane lipids. Studies with synthetic lipids (DeGier et al., 1968; Ladbrooke and Chapman, 1969; Seelig and Browning, 1978; Seelig and Waespe-Šarčevič, 1978) have indicated that these properties generally depend strongly on the lipid fatty acid composition. However, it must not be assumed a priori that any variations seen in membrane function in cells grown with avidin plus different exogenous fatty acids can be attributed entirely to the variations in lipid acyl chain composition, for it is possible that other aspects of the membrane composition (e.g., the membrane's content of various proteins or of lipids with various polar head-groups) could also vary depending on the fatty acid supplementation. Such secondary changes in membrane composition could in turn affect the membrane physical and functional properties. In this chapter, I have examined the characteristics of the membrane lipid phase transition in A. laidlawii B grown with avidin and various Class I (good growth substrate) fatty acids. As well, I have evaluated the extent to which

variations in the nature of the fatty acid supplement can alter the membrane composition (and thereby the lipid physical properties) in aspects other than the lipid acyl chains.

As I have noted earlier, a number of workers have made use of the ability of A. laidlawii B to incorporate substantial amounts of exogenous fatty acids, even in the absence of avidin, to manipulate the membrane lipid fatty acid composition. Studies using a variety of techniques, notably differential scanning calorimetry (DSC (Steim et al., 1969)), differential thermal analysis (DTA (McElhaney, 1974a, 1974b)), freeze-fracture electron microscopy (Verkleij et al., 1972; McElhaney, 1974b) and wide-angle X-ray diffraction (Engelman, 1971; Wallace and Engelman, 1978), have demonstrated that the midpoint temperature of the gel-to-liquid-crystalline phase transition in the membrane of this organism can be significantly altered by varying the exogenous fatty acid supplement(s). A few more subtle changes in the membrane lipid physical properties have also been detected upon altering the lipid fatty acyl composition. For example, the incorporation of high levels of isobranched fatty acids is found to prevent the normal aggregation of intramembrane particles (seen by freeze-fracture electron microscopy) at temperatures below the lipid phase transition range (Verkleij and Ververgaert, 1975). Curiously, both Butler et al. (1978) and Rottem et al. (1970) have found little difference in the ESR linewidth parameter $2T_{||}$ for a fatty acid spin label, 5-nitroxide palmitate, in A. laidlawii B membranes enriched in various fatty acids when the spectra are recorded at the growth temperature. As $2T_{||}$ is frequently used as an index of a spin label probe's mobility, and hence of membrane 'fluidity', it might seem that fatty acid changes are not a good way to change lipid 'fluidity'. However, other workers,

using different spin probes and measuring other motional parameters, have reported substantial differences in spectra recorded from membranes of different fatty acid composition (Tourtellotte et al., 1970; James and Branton, 1973). It seems safest to conclude, therefore, that changes in fatty acid composition may change some aspects of membrane lipid 'fluidity' more drastically than others. A full elucidation of the potential of the avidin/fatty acid culture technique for modifying membrane lipid properties in A. laidlawii B must await the development of a fuller description of the complex phenomenon of membrane lipid 'fluidity' itself.

The above-cited effects of exogenous fatty acid supplementation on A. laidlawii membrane properties combine a direct effect, that of altered lipid fatty acid composition on lipid physical properties, with a secondary effect, that of such other changes in membrane composition as may occur in the presence of an exogenous fatty acid on the properties of the membrane as a whole. A few studies have appeared in which the compositions of membrane components other than lipid acyl chains have been studied as a function of exogenous fatty acid supplementation. Pisetsky and Terry (1972) have reported only minor changes in the molecular weight distribution of membrane proteins (as determined by SDS-polyacrylamide gel electrophoresis [SDS-PAGE]) in cells grown with various saturated or cis-unsaturated fatty acids, and McElhaney et al. (1970) have reported that the lipid head-group distribution is unaltered by exogenous fatty acid supplementation. On the other hand, we have previously noted (Silvius et al., 1977) that certain exogenous fatty acids can significantly increase the membrane lipid/protein ratio. In general, membrane proteins in A. laidlawii B are felt to have little effect on the phase transition of the 'bulk' membrane lipids not directly bound to protein, since proteo-

lytically digested membranes, or protein-free dispersions of the extracted membrane lipids in water, exhibit thermal phase transitions and lipid spin-label mobilities very similar to those observed in native membranes (Steim et al., 1969; Rottem and Samuni, 1973). It is likely, however, that the integral membrane proteins bind a certain amount of lipid and strongly alter the properties of this 'boundary' lipid fraction from those of the free bilayer ('bulk') lipid, as has been observed for other integral proteins such as cytochrome c oxidase and the sarcoplasmic reticular ATPase (Jost et al., 1973, 1977; Hesketh et al., 1976). In fact, Butler et al. (1978) have recently observed that a portion of a spin-labelled fatty acid probe added to A. laidlawii B membranes is strongly immobilized on the ESR time scale (∿μsec) and may represent a 'boundary lipid' fraction. The possible effects of changes in the membrane lipid head-group composition on the lipid physical properties in A. laidlawii B have not been systematically investigated, but both De Kruijff et al. (1973) and Wieslander et al. (1978) have noted significant differences in the physical properties of various individual glycerolipid species from this organism. The possibility that changes in lipid head-group composition accompanying changes in the lipid fatty acid composition could contribute to any observed changes in the lipid physical properties is thus a real one.

In this chapter, I have used two approaches to assess the direct (lipid fatty acyl group-related) and indirect (lipid head-group- and protein-related) effects of avidin/fatty acid treatment on the properties of the A. laidlawii B membrane. First, I have investigated the effects of such treatment on the membrane lipid and protein composition. I have determined the membrane protein molecular weight distribution (by urea/

SDS-PAGE), the membrane lipid/protein ratio, and the relative proportions of the various membrane glycerolipids, as a function of the presence or absence of avidin and of the nature of the exogenous (Class I) fatty acid supplement used when cells are grown in the presence of avidin. Secondly, I have studied the thermotropic phase transitions of 'fatty acid homogeneous' membranes or lipids from A. laidlawii B under various conditions in order to determine how avidin/fatty acid-induced changes in the lipid fatty acyl composition, or in the membrane lipid head-group or protein composition, can affect the physical properties of the membrane lipids.

The method used to monitor thermotropic lipid phase transitions in the experiments described in this and the next chapter is differential thermal analysis (DTA). In this technique, two small cells, one containing an 'inert' thermal reference of fairly constant heat capacity and the other containing the sample of interest, are placed in contact with a large thermal reservoir of high thermal conductivity (a massive silver block) whose temperature is linearly varied with time. The temperature of each cell lags slightly behind that of the reservoir, the magnitude of the lag depending on the thermal conductivity, mass distribution and heat capacity of the cell and the material in it. The temperature difference $\delta(T)$ between the reference and sample cells will normally vary only slowly with the sample temperature T, but if the sample undergoes a thermotropic phase transition, the temperature lag in the sample cell will be transiently increased, and a trace of $\delta(T)$ vs. T will show a pronounced extremum (minimum for an endotherm, maximum for an exotherm) at the phase transition temperature. If the sample is heterogeneous and exhibits a broad phase transition (sometimes called a 'lateral phase separation'), the progress of the transition can be monitored by the deflec-

tion of the $\delta(T)$-vs.-T trace from its normally roughly linear baseline. DTA can thus be used to determine the peak or midpoint temperature and the temperature range of a lipid phase transition. Unfortunately, $\delta(T)$ can not be readily related to the absolute differential heat absorption by the sample, so absolute enthalpies of transition cannot be reliably determined by DTA. Enthalpies of transition can be measured, however, by the related technique of differential scanning calorimetry, in which the differential heat absorption by the sample is directly monitored. By monitoring the thermotropic phase transitions of membrane lipids by DTA, one can draw conclusions which are at least qualitatively correct regarding the effects of a variety of factors on the lipid physical behavior, just as the analysis of the phase transitions of other systems (e.g., of the melting properties of systems of mixed composition) can yield much information regarding the interactions of their component molecules.

Experimental Results and Implications

Lipid and Protein Composition - Three features of the membrane lipid and protein composition were studied as a function of avidin and fatty acid supplementation: the relative amounts of the major polar lipid head-group classes, the ratio of membrane lipid to membrane protein, and the molecular weight distribution of membrane proteins. The fatty acid supplements used in these experiments were all Class I species (good growth substrates for A. laidlawii B in the presence of avidin), as it is these species which can be most readily used to produce 'fatty acid-homogeneous' membranes from A. laidlawii B.

To study the effect of fatty acid supplementation on the lipid

head-group distribution, cells were grown to late log phase on various avidin/fatty acid-supplemented media, then harvested and the lipids extracted and freed of nonlipid contaminants by the procedure of Bligh and Dyer (1959) followed by silicic acid column chromatography as described in Chapter 2. The total lipid extracts were then spotted on silica gel G plates, which were developed with chloroform/methanol/water (70:25:5, v/v/v), sprayed with 3% CrO_3 in 60% H_2SO_4, and heated to visualize the separated lipid species as brown-to-black spots whose intensities are a rough indicator of the relative amounts of the various lipids present in the sample. The various lipids were identified by standard diagnostic reagents (ninhydrin for amino groups, phosphomolybdate reagent for phosphate, and anthrone for sugars (Christie, 1973)) and by comparison of their mobilities with those of known standards, which were either synthetic or previously purified from A. laidlawii B in our laboratory. The appearance of a typical developed chromatoplate is shown in Figure 6, where the identities of the major spots are given. The predominant lipid species are two neutral glycolipids, mono- and diglucosyl diglyceride (MGDG and DGDG), and an anionic phospholipid, phosphatidylglycerol (PG). Two other phosphate-containing lipids, glycerophosphoryl diglucosyl diglyceride (GPDGDG) and 3'-O-aminoacyl (mainly 3'-O-alanyl) esters of PG (O-PG), occur in lesser and variable amounts in the membrane as well. The structures of these five lipid species are shown in Figure 7. Other lipid species which occur in the A. laidlawii B membrane in trace amounts have been variously identified by various workers (Smith, 1971; Wieslander and Rilfors, 1977) and shall not concern us here, for they constitute a very small fraction of the total membrane lipids. In Plate I are shown a series of developed and charred chromatograms of lipid

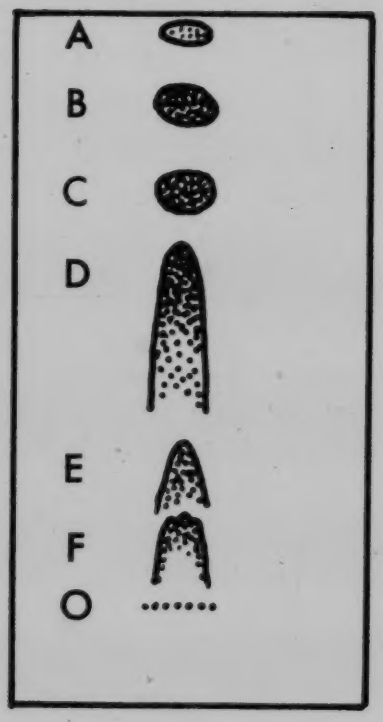


Figure 6. A representative chromatoplate for total A. laidlawii B membrane lipids, developed using the solvent chloroform/methanol/water (70:25:4, v/v/v) and silica gel H (0.5 mm thick) as the adsorbent, and charred after spraying with 3% CrO_3 in 60% sulfuric acid. The identities of the spots are: A, nonpolar lipids; B, monoglucosyl diglyceride; C, diglucosyl diglyceride; D, phosphatidylglycerol; E, glycerophosphoryl diglucosyl diglyceride; F, 3'-O-aminoacyl (mainly 3'-O-alanyl) phosphatidylglycerol.

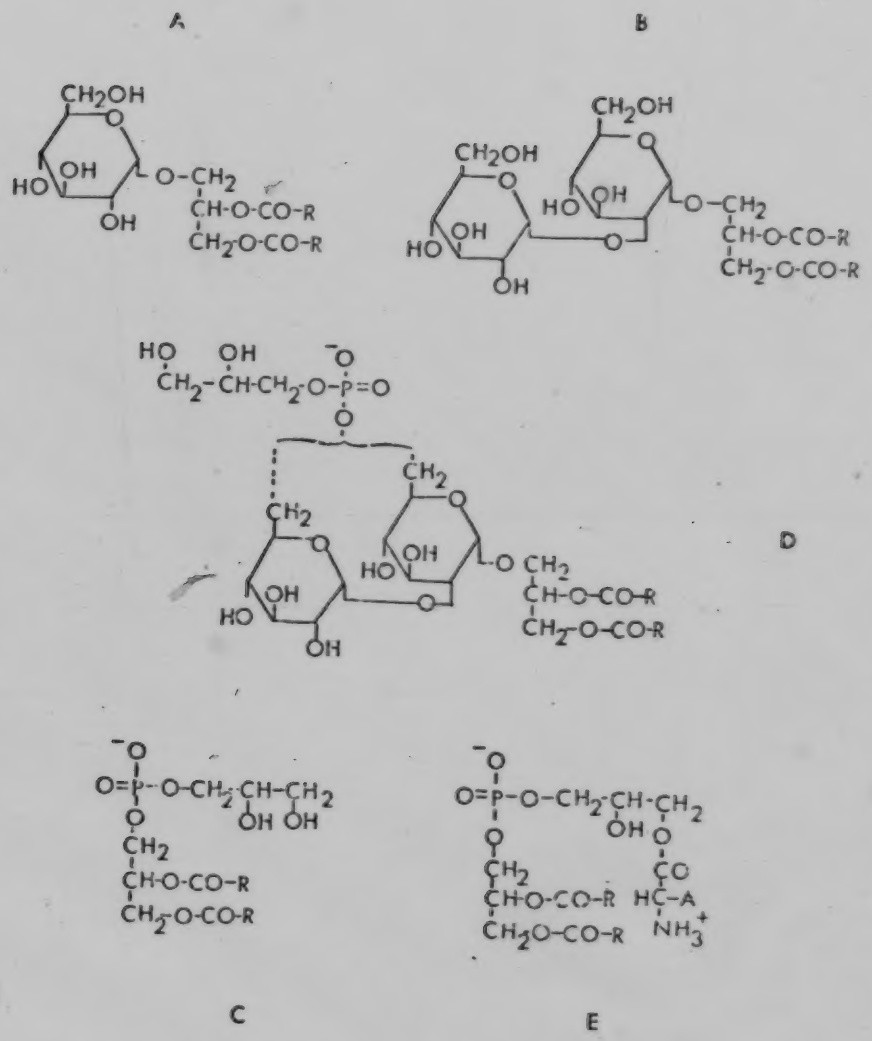
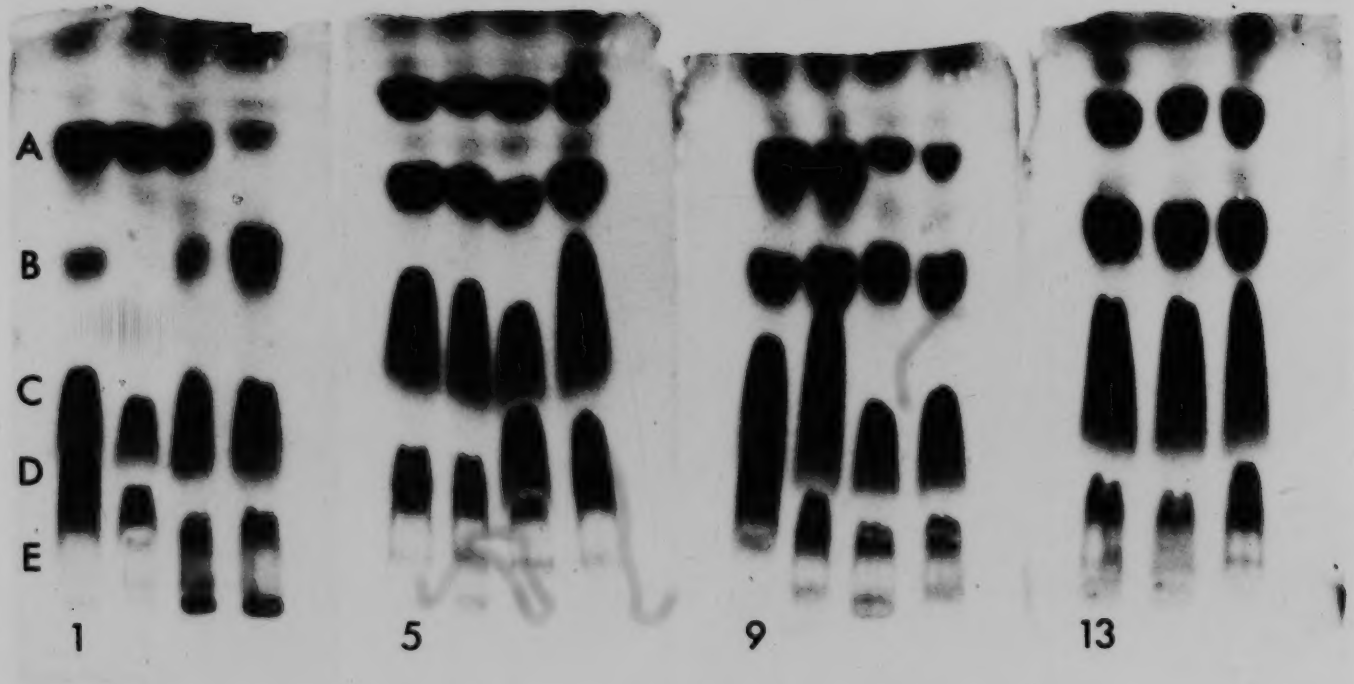


Figure 7. The chemical structures of the five major membrane polar lipids of A. laidlawii B. The structures shown are: A, monoglucosyl diglyceride; B, diglucosyl diglyceride; C, phosphatidylglycerol; D, glycerophosphoryl diglucosyl diglyceride; E, 3'-O-aminoacyl phosphatidylglycerol.

Plate 1. Developed and charred chromatoplates showing the lipid head-group distribution in various fatty acid-homogeneous A. laidlawii B membranes. Samples were prepared and TLC carried out as described in the text. The lipid spots are: A, monoglucosyl diglyceride; B, diglucosyl diglyceride; C, phosphatidylglycerol; D, glycerophosphoryl diglucosyl diglyceride; E, O-amino acyl phosphatidylglycerol. The samples run were from membranes of cells grown with avidin plus the following (in the shorthand nomenclature): 1-4, 14:0i, 15:0i, 16:0i, 17:0i; 5-8, 18:1tΔ^9, 18:1tΔ^{11}, 16:1tΔ^9, 18:1tΔ^6; 9-12, dl 15:0ai, dl 17:0ai, dl 19cp,tΔ^9, dl 19cp,tΔ^{11}; and 13-15, 18:1cΔ^4, 18:1cΔ^{11}, 18:1cΔ^{15}.


A
B
C
D
E
1
5
9
13

extracts of cells grown with avidin plus a variety of Class I fatty acids. It can be seen that the lipid head-group distribution is influenced by the nature of the exogenous fatty acid supplement, changing most notably when branched-chain fatty acids are used. McElhaney et al. (1970) have previously reported that exogenous fatty acid supplements do not alter the lipid head-group distribution in cells grown in the absence of avidin, but these workers did not use any branched-chain fatty acids in their studies. The two most dramatic effects of fatty acid supplementation on the proportions of the various lipid species are the marked enhancement of GPDGDG levels in the presence of shorter-chain fatty acids (14:0i, 15:0ai and 16:1tΔ^9) and the marked reduction of DGDG in the presence of the shorter-chain isobranched fatty acids. To more quantitatively evaluate the changes in lipid head-group proportions which result from variations in fatty acid supplementation, larger quantities of lipids from certain cultures of cells grown with avidin and a single fatty acid were fractionated by TLC as described above but without charring the plates. The major lipid species, MGDG, DGDG and PG, were recovered from the chromatoplates by scraping off the appropriate portions of the gel and eluting the lipids from the gel with methanol-chloroform (9:1, v/v). The relative amounts of these three lipids in a given sample were then determined by gas-liquid chromatography (GLC) of the methyl esters derived from each lipid in the presence of known levels of an internal standard fatty acid, which was included to calibrate the mass response of the GLC detection system. The results of a series of experiments using several Class I fatty acids are summarized in Table 7. It is clear that substantial variations in the ratios of the three major lipid species occur when A. laidlawii B is grown with avidin plus

Table 7

Molar Ratios of the Major Polar Lipid Species in A. laidlawii B Membranes from Cells Grown with Avidin (2 mg/l) and Various Class I Fatty Acids (0.12 mM)

Fatty Acid Supplement	MGDG/DGDG (±S.E.M.)	(MGDG + DGDG)/PG (±S.E.M.)
14:0i	18.1±3.5	2.7±1.4
16:0i	5.6±0.4	3.7±0.4
dl 15:0ai	4.8±1.1	3.9±0.5
dl 17:0ai	2.4±0.9	2.4±0.8
18:1tΔ^9	0.82±0.16	2.9±0.5
18:1cΔ^4	0.54±0.06[a]	2.3±0.2[a]
18:1cΔ^{11}	0.56±0.16	3.1±0.6
19:cp,tΔ^9	0.15±0.03[a]	2.1±0.1[a]
None (- avidin)	2.3±0.3	3.6±0.3

[a]The indicated errors for these samples represent the uncertainty for duplicate determinations of the lipid ratios in the same culture of cells. Because very limited quantities of these fatty acids were available, it was not possible to grow multiple cultures of the size needed for a full lipid analysis.

various fatty acids, with the greatest variability arising in the ratio of MGDG to DGDG. By contrast, the ratio of (MGDG + DGDG) to PG varies much less strongly with fatty acid composition. These results are readily explainable if MGDG and DGDG are derived from a common precursor, as is apparently the case for A. laidlawii (Smith, 1970), and if DGDG is derived from MGDG by a process that is sensitive to the properties of the membrane lipids, as Wieslander (Wieslander and Rilfors, 1977; Christiansson and Wieslander, 1978) has suggested for the A strain of A. laidlawii. It would appear, however, that the MGDG-to-DGDG conversion is not solely determined by lipid 'fluidity', as these writers have suggested, for there is always a marked elevation of MGDG levels over DGDG levels in the presence of branched-chain fatty acids, and a relative increase in DGDG levels in the presence of unsaturated and cyclopropane fatty acids, regardless of whether the phase transition temperatures of membrane lipids enriched in such fatty acids (vide infra) are high or low. In general, my results indicate that the major effect of changes in the exogenous fatty acid supplement on the lipid head-group composition will be a change in the balance of the two neutral glycolipids, MGDG and DGDG, with a much less drastic change in the ratio of neutral glycolipids to PG (and, judging from the results shown in Plate I, in the ratio of neutral glycolipids to phosphate-containing lipids in general). The probable physical-chemical consequences of these changes in lipid head-group composition will be evaluated in a later section.

I next turned my study of fatty acid supplementation effects on membrane composition to a consideration of the membrane protein composition, for proteins not only mediate many membrane-related processes which are of biochemical interest but can also affect the physical pro-

perties of the membrane lipids (Jost et al., 1973, 1977; Hesketh et al., 1976). I first determined the ratio of total membrane-associated protein to membrane lipid. The Lowry protein assay as modified by Hartree (1972) was used to determine protein, and analytical GLC of lipid-derived methyl esters in the presence of an internal standard (to calibrate the detector mass response) was used to determine the μmoles of lipid acyl chains (and of diacyl lipids) in a sample of known protein content, isolating the lipids from the sample as described above. The results of these experiments are shown in Table 8, where the effects of a representative set of Class I fatty acids have been investigated. All of the fatty acid supplements used elevated the membrane lipid/protein ratio of cells grown without avidin or fatty acid supplementation. However, trans-unsaturated or -cyclopropyl fatty acids elevated this ratio only slightly, while branched-chain fatty acids did so more strongly and cis-unsaturated species did so most strongly, almost doubling the relative proportion of lipids in the membrane. The observed lipid/protein ratios in avidin/fatty acid-grown cells generally lie in the range of values previously reported for cells grown with various fatty acids in the absence of avidin (Silvius et al., 1977), suggesting that avidin itself does not significantly alter the relative rates of membrane protein and diacyl glycerolipid synthesis in vivo. There is no obvious general relationship between the membrane lipid/protein ratio and the physical properties (e.g., the phase transition temperatures) of the lipids in cells cultured with avidin plus various fatty acids. However, the data of Table 8 suggest that the lipid/protein ratio is sensitive to certain features of the lipid acyl chain structure (e.g., the presence of a cis- double bond) which may be important in determining the rate of utilization of fatty acids or

Table 8

Membrane Lipid/Protein Ratios for Cells Grown with Avidin (2 mg/l) and Various Class I Fatty Acids (0.12 mM)

Fatty Acid Supplement	Lipid/Protein (umoles/mg) ± S.E.M.
14:0i	.83 ± .07
15:0i	.79 ± .06
16:0i	.77 ± .11
17:0i	.88 ± .08
dl 15:0ai	.84 ± .06
dl 17:0ai	.70 + .11
$16:1t\Delta^9$	.57 ± .03
$18:1t\Delta^9$	.68 ± .01
$18:1t\Delta^{11}$	.63 ± .05
$18:1c\Delta^4$	.98 ± .07
$18:1c\Delta^{11}$	1.01 ± .08
$19:cp,t\Delta^9$	.57 ± .03
None (- avidin)	.56 ± .08
16:0 (- avidin)	.75 ± .14

[a]Lipid/protein ratios given were determined as described in the text, and are the averages of values determined in at least two separate experiments.

fatty acyl derivatives for some key step in membrane lipid synthesis. To return to a more practical level, it is expected on the basis of these data that cells grown on avidin plus different fatty acids may differ in their relative amounts of 'bulk' and protein-associated lipids (Steim et al., 1969; Butler et al., 1978), a fact which may be important in the interpretation of certain types of studies of membrane lipid dynamic properties in particular.

To test the possibility that certain proteins might become selectively enriched or depleted in the membranes of cells grown with avidin and certain fatty acids, small samples of such membranes were subjected to polyacrylamide gel electrophoresis in the presence of urea (6 M) and SDS (0.15%) under reducing conditions (Zahler, 1974), then stained with Coomassie blue and destained by the procedure of Weber and Osborn (1969). The details of the electrophoresis, Coomassie blue staining, and spectrophotometric scanning of the gels have been described in Chapter 2. Representative gel scans (at 596 nm) are shown in Figure 8 for the total membrane-associated proteins of cells grown with elaidic acid ($18{:}1t\Delta^{9}$) with or without avidin, with isopalmitic acid (16:0i) plus avidin, and with anteisopentadecanoic acid (15:0ai) plus avidin. While small differences in the Coomassie blue staining profiles can be seen for the various preparations, the protein molecular weight distributions are generally quite similar regardless of what fatty acid supplement was used or whether or not avidin was present in the culture medium. In fact, different preparations of membranes from multiple cultures of cells grown under the same conditions often showed variations in their protein composition which were as great as those shown in Figure 8. Therefore, while it is certainly not reasonable to assume a priori that

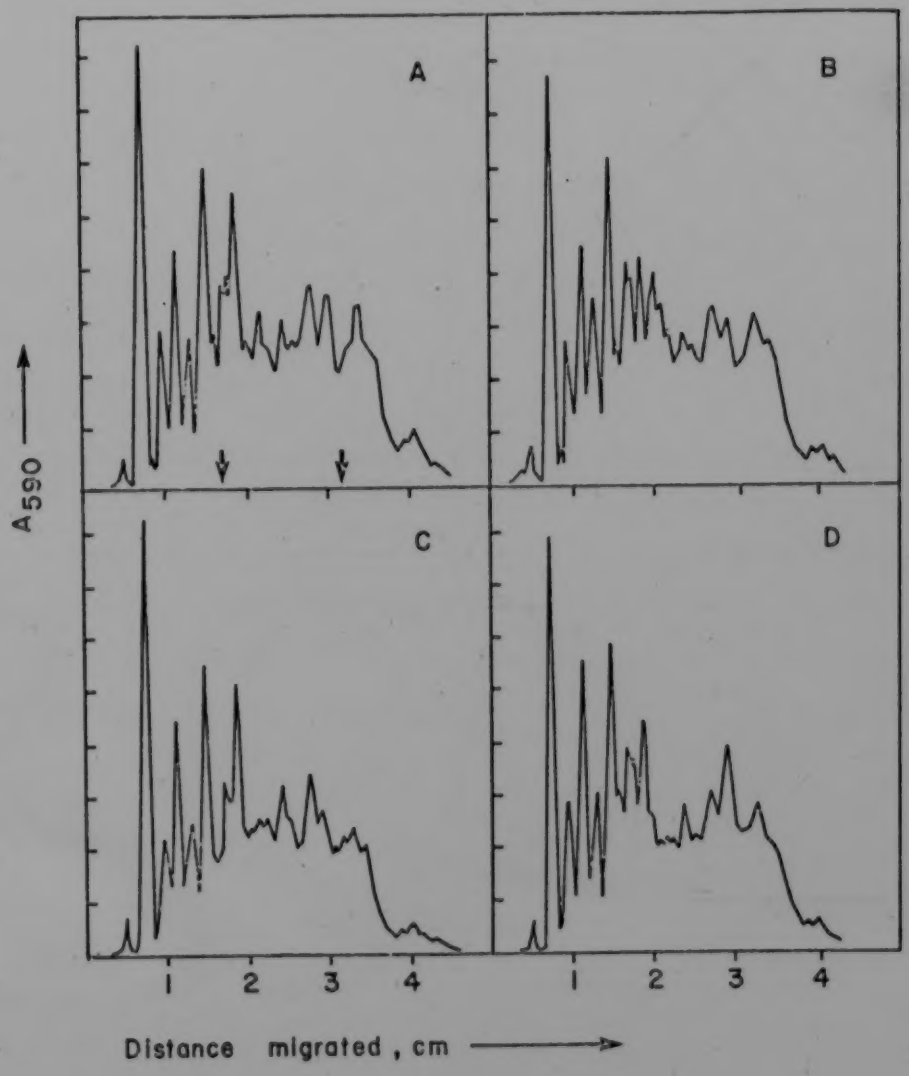


Figure 8. Coomassie blue staining profiles for SDS-polyacrylamide gels of A. laidlawii B membrane proteins. The membrane samples shown are from: A, elaidic acid-grown cells; B, elaidic acid/avidin-grown cells; C, isopalmitic acid/avidin-grown cells; D, anteisopentadecanoic acid/avidin-grown cells. The first and second arrows in panel A represent the distances migrated by bovine serum albumin (mol. wt. 63K) and DNase I (mol. wt. 31K), respectively.

the level of any one membrane protein is independent of the membrane fatty acid composition or of the presence or absence of avidin, it is clear that, in general, the relative rates of synthesis of various membrane proteins are not substantially altered by growth of A. laidlawii B on various Class I fatty acids plus avidin. This conclusion is in agreement with the findings of Pisetsky and Terry (1972), who used SDS-PAGE to study the protein molecular weight distribution in the membranes of cells of this organism grown with various fatty acids in the absence of avidin.

Lipid Phase Transitions of Membranes or Isolated Membrane Components from Avidin/Fatty Acid-Supplemented Cells - My studies of the lipid phase transitions of A. laidlawii B membranes from 'fatty acid-homogeneous' cells began with an investigation of the contribution of various normal membrane components to the overall membrane phase transition. These experiments were mostly carried out using cells grown in media supplemented with avidin plus isopalmitate (16:0i) or elaidate ($18:1t\Delta^9$).

One set of experiments focused on the role of membrane components other than glycerolipids in modifying the overall membrane phase transition behavior. The following preparations were derived from isopalmitate- or elaidate-homogeneous A. laidlawii B cells: (i), whole membranes; (ii), a total lipid extract; (iii), a membrane polar lipid extract, freed of nonpolar lipids by discarding the chloroform eluate from a column of Bio-Sil to which the total extracted lipids had been applied (see Chapter 2); and membrane polar lipid extracts equlibrated with solutions of either (iv) 20 mM $MgCl_2$ or (v) 50 mM EDTA according to the procedure of Folch et al. (1957). Comparison of the phase transitions determined by DTA for appropriate combinations of the above samples (lyophilized

from benzene and rehydrated with 50% (w/w) water in the case of lipid extracts, as described in Chapter 2) allows us to determine the effect of membrane protein ((i) vs. (ii)), membrane nonpolar lipids ((ii) vs. (iii)) or the membrane divalent cation content ((iv) vs. (v)) on the overall membrane phase transition. The results of one such set of experiments, for isopalmitate-homogeneous membranes, are shown in Figure 9. While the transition endotherm for the isolated membranes is smaller than that for the isolated lipids (a consequence of the fact that the membrane pellet obtained by centrifugation contains only one-tenth to one-fifth of the lipid per unit volume that the hydrated lipid dispersion does), it is clearly similar to the transition endotherm for the extracted lipids. This finding was typical in my experiments and agrees with previous results (Steim et al., 1969; McElhaney, 1974a) using A. laidlawii B cells which were not fatty acid-homogeneous. Therefore, membrane protein does not seem to significantly modify the phase transition properties of the 'bulk' membrane lipid phase, although it almost certainly sequesters some lipid (probably on the order of 10-25% of the total lipid (Steim et al., 1969)) as 'boundary' lipid incapable of taking part in the bulk lipid phase transition (Hesketh et al., 1976).

Examples of the effects of two other normal membrane components, nonpolar lipids (mainly carotenoids) and divalent cations (represented here by Mg^{2+}, normally the major membrane-associated divalent cation (Kahane et al., 1973)), on the lipid phase transition are also shown in Figure 9. Lipid extracts containing carotenoids (isolated under a nitrogen atmosphere and in semidarkness to minimize carotenoid degradation) sometimes showed a slightly broader phase transition than did pure polar lipid extracts, but in general the overall characteristics of the transi-

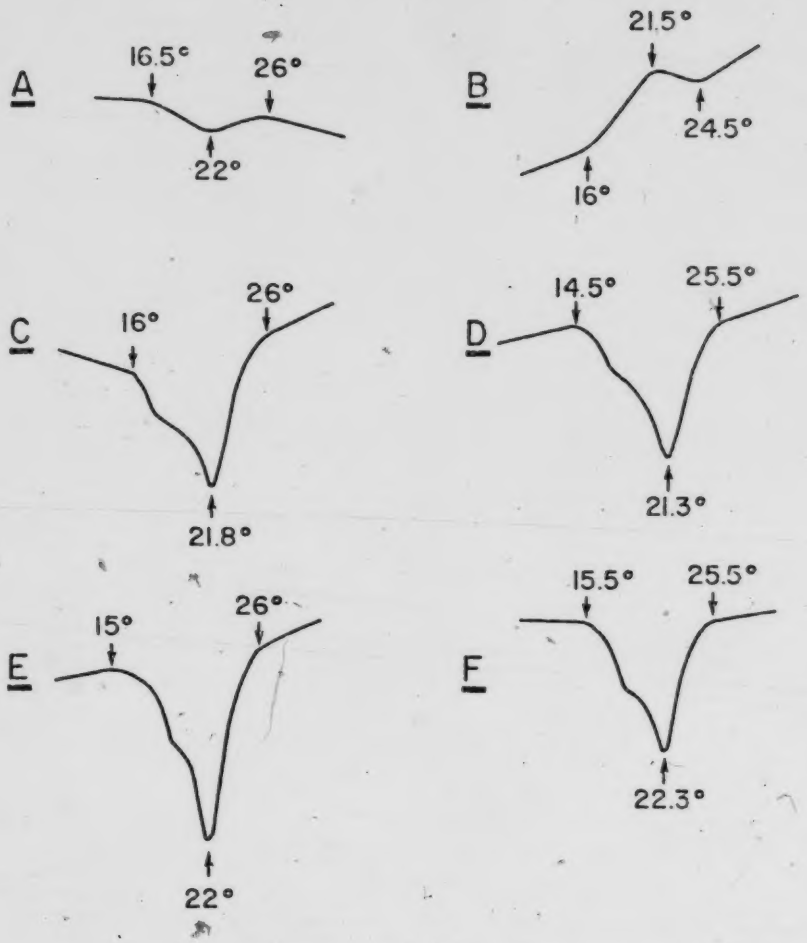


Figure 9. Representative thermograms determined by DTA of isopalmitate-homogeneous A. laidlawii B membranes or membrane components. All samples were analyzed at scan rates of 5°/min and were prepared as described in the text. The samples are designated in the figure as follows: A, intact membranes (heating); B, intact membranes (cooling); C, total membrane lipids; D, total membrane polar lipids; E, total polar lipids equilibrated with 50 mM $MgCl_2$; F, total lipids equilibrated with 50 mM EDTA.

tion endotherm were quite similar in the presence and absence of carotenoids. The lipid extracts equilibrated with $MgCl_2$ or with EDTA solutions showed quite similar endothermic transition profiles by DTA. This result is somewhat surprising in view of the substantial membrane content of anionic lipids (phosphatidylglycerol and glycerophosphoryldiglycosyldiglyceride), which are known to show a significant dependence of their phase properties on the levels of divalent cations associated with them (Papahadjopoulos et al., 1975; Van Dijck et al., 1978; Findlay and Barton, 1978). It would appear that the electrically neutral lipid species in the A. laidlawii B membrane dilute out the negatively charged species so that the normally complex phase behavior of the latter in the presence of divalent cations is no longer observed. A similar result has in fact been reported by Findlay and Barton (1978), who studied the effects of Ca^{2+} and Mg^{2+} on synthetic phosphatidylglycerols (PG's) and phosphatidylglycerol/phosphatidylcholine (PG/PC) mixtures. These workers found that the complex thermotropic behavior of pure saturated PG's which was observed in the presence of divalent cations reverted to a much simpler behavior in the presence of even small amounts of PC's. Specifically, the multiple endotherms observed on heating pure Ca^{2+}-PG's or Mg^{2+}-PG's were replaced by a single sharp endotherm at a lower temperature when as little as 5 mole percent of the PC species of like fatty acid composition was also present. In this regard, it is interesting to note that phosphatidylserine decarboxylase mutants of E. coli can accumulate very high levels (>50%) of phosphatidylserine in their membranes in the presence of magnesium yet still remain viable (Hawrot and Kennedy, 1978), while pure PS does not form stable hydrated bilayers in the presence of divalent cations (Papahadjopoulos et al., 1975; Hauser et al., 1977).

The results presented so far in this section indicate that even if variations in the levels of membrane proteins or carotenoids do occur when cells are grown with avidin plus various fatty acids, such variations will not result in any major changes in the thermotropic properties of the 'bulk' membrane lipid phase at least. Many of the most important microscopic features of lipid-lipid interactions, such as the energy of lateral acyl chain association, the strength of intermolecular coupling (i.e., the cooperativity), and the mixing properties of the various lipid species, all bear on and are reflected in the nature of the lipid phase transition. Therefore, the very modest observed effects of membrane proteins or carotenoids on the polar lipid phase transition suggest that these former membrane components have only a modest effect on the 'bulk' lipid physical properties in general. To evaluate the effects of variations in the proportions of various lipid head-group species on the membrane lipid properties, I again studied the thermotropic behavior of various lipids and lipid mixtures by DTA. To do this, I isolated various lipid fractions (single pure lipids, total phospholipids, total neutral glycolipids, etc.) from A. laidlawii B cells made homogeneous in isopalmitate (16:0i) or elaidate ($18:1t\Delta^9$) using standard column and thin-layer chromatographic procedures, as described in Chapter 2. The separate lipid fractions were then rehydrated and their phase transitions monitored by DTA in the usual fashion.

Heating scans for several lipid fractions derived from cells grown with isopalmitate and avidin are shown in Figure 10; the thermotropic behavior of the corresponding elaidate-containing lipids was similar in all respects. Of the three major membrane lipid species, MGDG, DGDG and PG (see Figure 7 for structures), PG shows a thermotropic

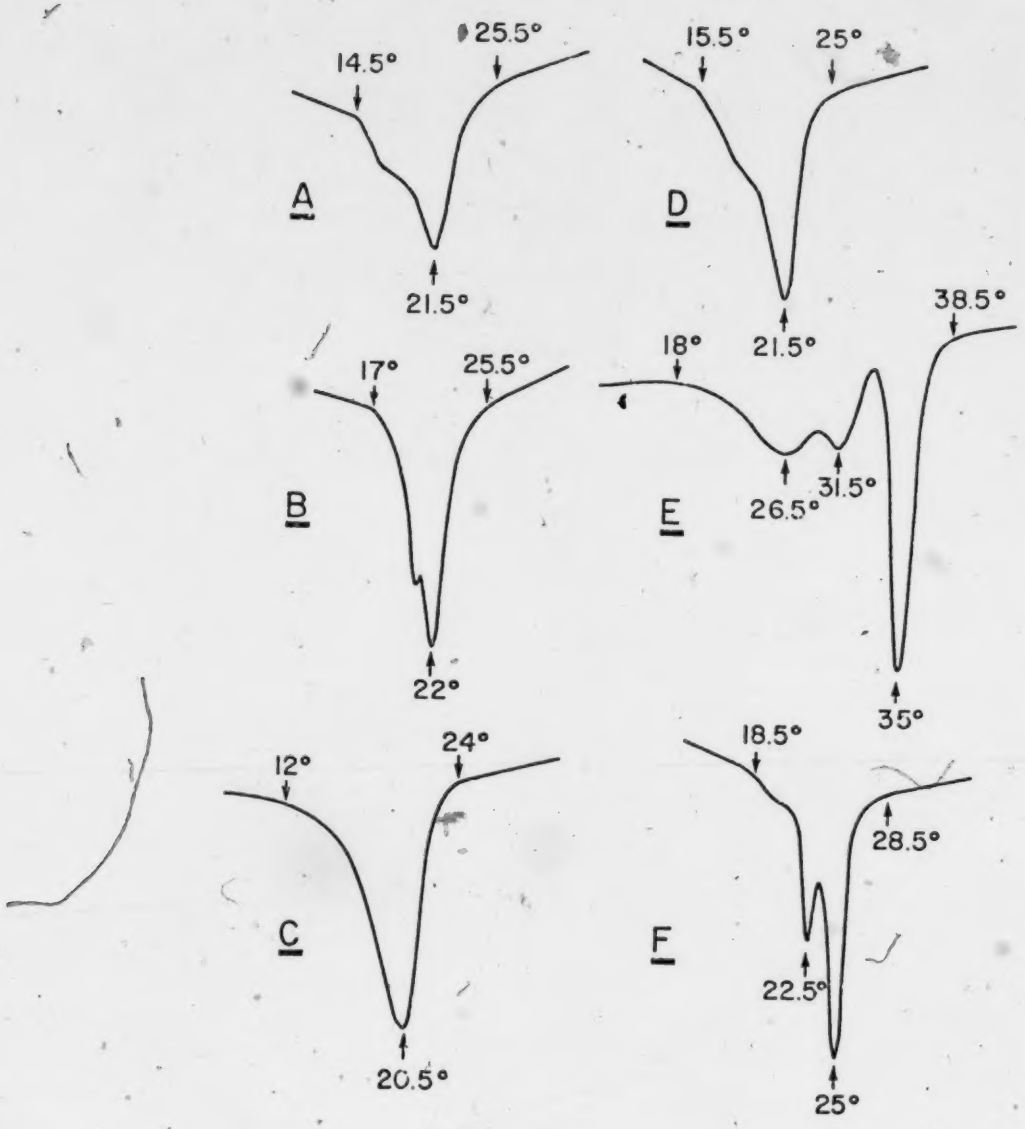


Figure 10. Representative thermograms for various membrane lipid fractions from isopalmitate-homogeneous A. laidlawii B membranes, isolated and prepared for DTA as described in the text. The samples are designated by the letters: A, total membrane polar lipids; B, total neutral glycolipids; C, total phosphate-containing lipids; D, phosphatidylglycerol; E, diglucosyl diglyceride; and F, monoglucosyl diglyceride. All samples were heated at 5°/min to give the thermograms shown.

behavior quite similar to that of the total membrane lipids. The total phosphorylated lipid fraction shows a thermotropic behavior similar to that of pure PG, which is the major component of this fraction. As pure MGDG readily forms nonbilayer phases at physiological temperatures, most notably a hexagonal-II phase in which the lipid molecules are arranged in long cylindrical arrays with the lipid head-groups lying near the centers of the cylinders (Wieslander et al., 1978), it is perhaps surprising that this lipid upon heating exhibits a strongly endothermic transition near the transition temperature of the total membrane lipids. Hydrated MGDG derived from 'elaidate-homogeneous' cells exhibits a similar thermotropic behavior, in agreement with the results of Wieslander et al. (1978). Therefore, it would seem that the interactions of the lipid acyl chains in the nonlamellar phase formed by pure MGDG in the physiological temperature range are basically similar to those of the acyl chains of other glycerolipids which form lamellar phases in this temperature region. The thermotropic behavior of pure DGDG is rather complex, with a series of endotherms observable on heating and a marked and quite reproducible supercooling to give a single exothermic transition (at 15.5°C for diisopalmitoyl DGDG) upon subsequent cooling. De Kruijff et al. (1973) and Wieslander et al. (1978) have also noted this rather complex phase behavior, yet the latter authors have concluded that DGDG persists in a lamellar arrangement throughout the temperature range studied. When the total neutral glycolipids (MGDG and DGDG, plus a small amount of an unidentified lipid of intermediate polarity under standard TLC conditions) are hydrated and studied by DTA, a fairly sharp, reversible transition replaces the rather complex transitions seen for the individual glycolipids. The midpoint temperature of the neutral

glycolipid transition, 22°C, is very similar to that of the total membrane lipids, 21°C, or that of the PG fraction, 21.5°C. Therefore, it seems that if one neglects some rather exotic phase behavior seen only with the pure forms of the glycolipids MGDG and DGDG, the major components of the lipid mixture found in the A. laidlawii B membrane can intermix fairly easily, with fairly similar like-like and like-unlike molecular interaction, and should exhibit little tendency to segregate out in lipid domains of composition drastically differing from that of the overall membrane lipid pool. Fairly modest changes in the relative proportions of the various lipid head-group classes, such as those resulting from the use of various fatty acids as cell growth substrates in the presence of avidin (vide supra), should not lead to major changes in the overall physical properties of the membrane lipids beyond those which would be observed if the fatty acid composition were changed without any accompanying changes in the lipid head-group composition. However, the differences observed in the T_c values of the different isolated lipid species suggest that the overall membrane lipid T_c value could be significantly dependent on the proportions of the various lipid head-group classes as well as on the fatty acid composition. Furthermore, one might expect that membranes containing greater amounts of MGDG might more readily form small regions of nonlamellar structure, at least transiently, and might therefore show an enhancement of the rate of certain membrane processes, such as fusion, or lipid-transbilayer 'flip-flop', which may proceed by the formation of nonlamellar lipid phases (Cullis and de Kruijff, 1976; Cullis and Hope, 1978). In general, however, the effects of lipid head-group changes on the membrane lipid properties will likely be much less profound than the effects due to altered lipid

fatty acyl composition when cells are grown with avidin plus different Class I fatty acids.

Once I had identified the possible influences on the membrane lipid phase transition of various changes in membrane composition other than those of the lipid fatty acid chains, I turned my attention to the effect of the membrane lipid fatty acyl chain structure on the midpoint temperature and temperature range of the lipid phase transition under conditions of fatty acid homogeneity. A. laidlawii B was grown in the presence of avidin and various single Class I fatty acids (see Table 3), and the cells were harvested in late log phase, washed once, and the total cell lipids extracted, purified and prepared for differential thermal analysis (DTA) as described in Chapter 2. Representative thermograms are shown in Figure 11, where a thermogram for the lipids from cells grown with palmitic acid (16:0) but without avidin is shown as well. It should be noted that essentially all of the lipid in A. laidlawii B has been found in the cell membrane (Pollack et al., 1965b), and in fact in some experiments where isolated membranes rather than whole cells were extracted to obtain lipids for DTA, the measured thermograms were indistinguishable from those of lipids extracted from whole cells. Therefore, my assumption that whole washed cells provide a true membrane lipid fraction upon lipid extraction seems justified. Differential thermograms such as those shown in Figure 11 cannot readily be calibrated to yield absolute heats of transition, but the deviation of an endotherm from the baseline at a given temperature is roughly proportional to the differential heat capacity of the sample (Smothers and Chiang, 1966), which in turn would be expected to be roughly proportional to the amount of lipid undergoing a phase change over the infinitesimal temperature

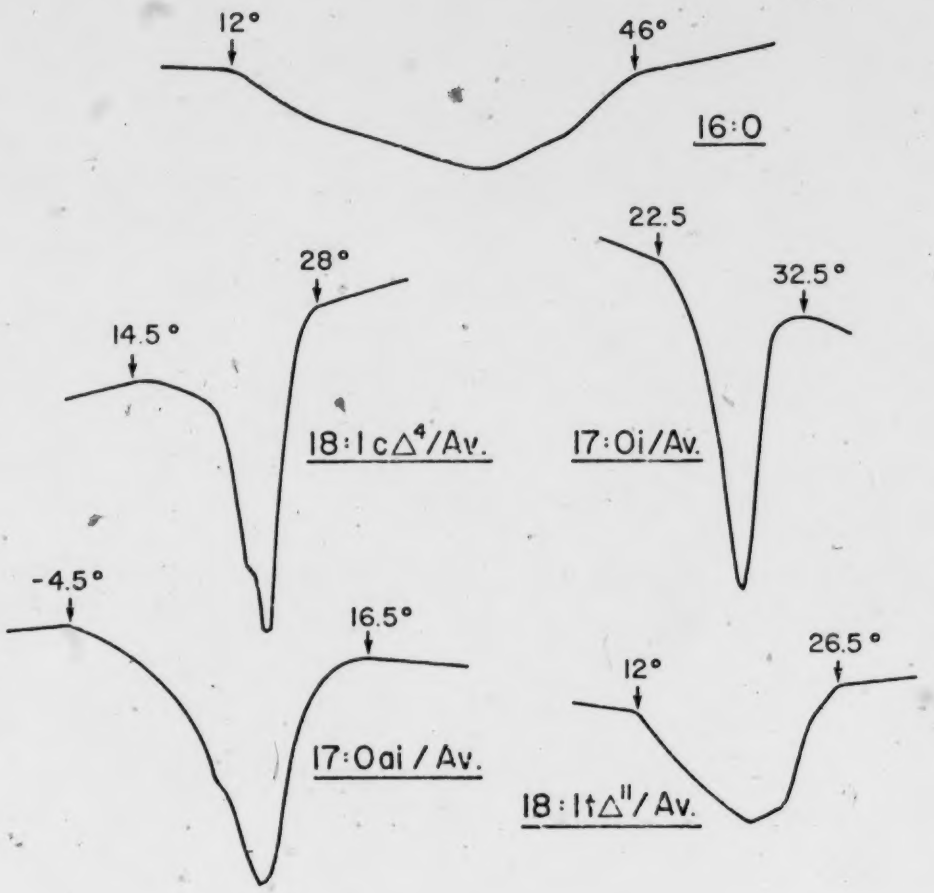


Figure 11. Representative heating thermograms for the total lipids of membranes of A. laidlawii B cultured with various fatty acids (whose symbols are given next to each thermogram) with or without avidin (the letters 'Av.' indicate that avidin was present). The total membrane lipids were extracted and prepared for DTA as described in the text. All samples were analyzed by DTA at a heating rate of 5°/min.

range centered around the temperature of interest. Therefore, integration of the endotherm from the onset of the transition to any temperature T_o should allow at least a rough determination of the proportion of gel-state and liquid-crystalline lipid present at T_o, according to the equation

$$F_{\ell c}(T_o) = \frac{\int_o^{T_o} \delta(T)\, dt}{\int_o^{\infty} \delta(T)\, dt} \simeq \frac{\int_{T_s}^{T_o} \delta(T)\, dt}{\int_{T_s}^{T_\ell} \delta(T)\, dT} \qquad [4\text{-}1]$$

where T is the absolute temperature, $\delta(T)$ is the magnitude of the deviation of the DTA trace from the baseline at temperature T, $F_{\ell c}(T_o)$ is the fraction of liquid-crystalline lipid at T_o, and T_s and T_ℓ are respectively the points at which the thermogram first shows a detectable deviation from, and appears to return to, the baseline. Using the above equation and integrating experimentally obtained DTA endotherms by means of a trapezoid rule approximation (Rodin, 1970) over 1° temperature intervals, I calculated the transition midpoints T_{AL} and the temperature ranges from 10% to 90% completion, $\Delta T(10\%\text{-}90\%)$, of the phase transitions of total lipids from cells grown with avidin and various Class I fatty acids or from control cultures grown without these supplements. These data are presented in Table 9, where it can be seen that the membrane lipid phase transition midpoint can be varied over a range of about 50° by using appropriate Class I fatty acids plus avidin to grow A. laidlawii B. This finding is not particularly novel, for such variations of T_{AL} can be obtained by varying the fatty acid supplement added to the culture medium even in the absence of avidin (McElhaney, 1974a). More significantly, the data of Table 9 indicate that the temperature

Table 9

Midpoint Temperatures (T_{AL}) and Transition Widths for the Gel-to-Liquid-Crystalline Phase Transition of Total A. laidlawii B Membrane Lipids Made Homogeneous in Various Fatty Acids

Fatty Acid	% of Total Fatty Acids	T_{AL}	$\delta T(10\rightarrow90)$ [a]
15:0	92	36.7°	10.6°
14:0i	96	10.1°	5.1°
15:0i	98	14.8°	9.0°
16:0i	99	21.8°	6.1°
17:0i	98	28.8°	4.8°
dl 14:0ai	95	-14.9°	7.6°
dl 15:0ai	95	-4.7°	8.3°
dl 16:0ai	100	4.1°	11.1°
dl 17:0ai	96	8.2°	9.4°
dl 18:0ai	99	21.8°	6.1°
16:1tΔ^9	98	7.6°	8.9°
18:1tΔ^6	96	22.2°	11.6°
18:1tΔ^9	98	20.1°	10.4°
18:1tΔ^{11}	99	20.0°	8.4°
18:1cΔ^4	91[b]	24.1°	5.0°
18:1cΔ^{11}	96	-8.3°	27.1°
18:1cΔ^{15}	91[b]	27.6°	8.9°
19:cp,tΔ^9	97	23.8°	10.9°
16:0/No Avidin	67	31.2°	17.8°
18:1tΔ^9/No Avidin	72	32.7°	12.9°
16:0i/No Avidin	76	25.8°	11.2°

[a] $\delta T(10\rightarrow90)$ is defined as the temperature range required for the transition to pass from 10% to 90% of completion.

[b] These fatty acids were only ∿ 93% pure as they were added to the culture medium.

range of the lipid phase transition can be dramatically narrowed, as compared with control values, by using an appropriate exogenous fatty acid and avidin. By contrast, the width of the phase transition is at best only slightly reduced with respect to control values when cells are grown with various fatty acids but without avidin (McElhaney, 1974). The narrowing of the lipid phase transition temperature range in cells grown with certain Class I fatty acids plus avidin is significant for two reasons. First, the sharpness of the lipid transition in fatty acid-homogeneous membranes suggests that the various major lipids in the membrane mix ideally or nearly ideally in both the gel and liquid-crystalline states, for otherwise much broader and probably bimodal thermograms should be observed. This conclusion is consistent with the results obtained from DTA of the individual lipid species from fatty acid-homogeneous cell membranes, which were presented earlier in this section. The second reason for the importance of the sharpened lipid phase transition in fatty acid-homogeneous cell membranes is a practical one: by use of the A. laidlawii/avidin/fatty acid system, it becomes possible to independently vary the sharpness and the position of the membrane lipid phase transition in a living cell over a wider range than has previously been possible. Most importantly, it is now possible to reduce the width, and to control the position, of the lipid phase transition to such an extent that one can observe the entire membrane phase transition, and its effects on membrane-associated processes, over a conveniently narrow range of temperatures lying entirely within the physiological temperature range (roughly 10°-40°C). The ability to vary the sharpness of the membrane lipid phase transition, as well as its position, over a substantial range of values has considerable potential usefulness in certain

types of studies, as we shall see later in my studies of a membrane-bound enzyme in A. laidlawii B (Chapter 7).

Conclusions

The results described in this chapter, taken together with those of the previous chapter, indicate that the A. laidlawii B/avidin/fatty acid system represents the most convenient and widely yet specifically manipulable native biological preparation yet reported in which one can study lipid physical properties and their relationship to membrane function. My data show that fatty acid-homogeneous membranes of A. laidlawii B can be prepared with lipid phase transition temperatures varying over a range as wide as that obtainable by culturing the organism with various fatty acids but without avidin (and hence with a heterogeneous fatty acid composition). The variations observed in membrane protein and lipid head-group composition when cultures are grown with various fatty acids and avidin are no more pronounced than others have observed in cultures which were cultured with various fatty acids but without avidin (Pisetsky and Terry, 1972; Wieslander and Rilfors, 1977). Furthermore, I have shown that these 'secondary' compositional changes by themselves are unlikely to greatly alter the physical properties of the membrane lipids in most respects, at least when Class I fatty acids are used as growth substrates. As we shall see in the next chapter, the transition midpoint temperatures (T_{AL}) for various preparations of fatty acid-homogeneous A. laidlawii B membrane lipids correlate very well with the transition temperatures in a lipid system of fixed head-group composition, the pure hydrated phosphatidylcholines, with the same acyl chains. This finding supports my conclusion that the effects of lipid head-group

variations on the A. laidlawii lipid transition temperature are slight. Most importantly, in this chapter I have shown that the lipid species of fatty acid-homogeneous membranes appear to mix ideally or nearly ideally, exhibiting similar phase transition temperatures when separated and rather sharp transitions when combined, so that the bulk lipid phase can be treated, to a first approximation at least, as a single homogeneous phase. The possibility of reducing the temperature range of the membrane lipid phase transition by over threefold from control values, using an appropriate exogenous fatty acid supplement plus avidin in the cell culture medium, allows the investigator to study a number of interesting phenomena related to the lipid phase transition in a biological membrane in which a large fraction of the lipids take part in the transition over a temperature range of a few degrees.

The findings reported in the last two chapters indicate that the potential of the A. laidlawii/avidin/fatty acid system for specific and controlled regulation of the lipid environment of membrane proteins in situ approaches in some respects the potential of the lipid-reconstitution method. Homogeneity of the lipid fatty acyl composition is easily obtained, the lipid phase transition midpoint temperature can be easily altered over a wide range, and in some cases (e.g., by using isoheptadecanoic acid), it is possible to obtain membranes whose lipid phase transition range width approaches that of a pure lipid species. However, the A. laidlawii B membrane remains highly heterogeneous in its protein composition (as a living cell presumably must) and contains lipids with several distinct head-groups even when it is homogeneous in fatty acid composition. My attempts to modify the lipid head-group composition in the absence of fatty acid changes by using glycerol, glycerol 3-phosphate

or ribose as possible carbon sources in place of glucose were wholly unsuccessful. However, it has been possible to modify some of the lipid head-groups in isolated A. laidlawii membranes by using phospholipases (Bevers et al., 1977), and other limited alterations (possibly even modest simplifications) in the lipid head-group distribution may be possible using this approach. Recent studies with E. coli mutants deficient in various steps in phospholipid metabolic pathways have indicated that the cells need not maintain a specific lipid head-group composition in order to remain viable (Raetz and Foulds, 1977; Hawrot and Kennedy, 1978; Pluschke et al., 1978), and this is probably also true for A. laidlawii B, as the changes that I observed in lipid head-group distributions with varying lipid fatty acid supplementation suggest. It thus may yet be possible to find a suitable inhibitor (of a glucosyltransferase, perhaps, or of an aminoacyltransferase presumably involved in O-PG synthesis) which will specifically block the synthesis of one or more of the glycerolipid species in the A. laidlawii B membrane, thus simplifying the overall lipid head-group composition. An encouraging precedent is the use of diphenylamine to specifically block carotenoid oxidation in this organism (Smith and Henrickson, 1966). A sober assessment of the current status of the A. laidlawii B system, however, suggests that the membrane of this organism is unlikely in the near future to be suitable for studies requiring complete homogeneity of either membrane proteins or lipid head-groups.

CHAPTER 5

CHEMICAL SYNTHESIS AND PHYSICAL PROPERTIES OF SOME NOVEL DIACYL PHOSPHATIDYLCHOLINES

Background

The advantages of the fatty acid-homogeneous A. laidlawii B membrane will undoubtedly be fully realized only if still simpler model membrane systems are available in order to resolve and clarify some of the features of the lipid behavior in the A. laidlawii system. One such model system would be the lamellar phase of a pure diacyl phospholipid dispersed in water. To date, the most intensively studied class of synthetic phospholipids has been the diacyl phosphatidylcholines (PC's) with fourteen- to eighteen-carbon acyl chains. These lipids have several properties which make them useful models for the study of membrane lipid behavior. Most notably, they readily form hydrated lamellar bilayer structures in water, which exhibit semipermeability properties similar to those of membrane lipids, they are readily synthesized, and they are chemically stable under most conditions of storage or assay (Papahadjopoulos, 1973; Bangham et al., 1974). Using straight-chain PC's of various chain lengths and degrees of unsaturation, the dependence of bilayer permeability to polar solutes and of the lipid gel-to-liquid-crystalline phase transition temperature on the structure of the lipid acyl chains has been investigated (DeGier et al., 1968; Ladbrooke and Chapman, 1969). These studies have shown that a decrease in the acyl chain length, or an increase in the extent of unsaturation (cis-double bonds have more effect than trans-olefinic moieties) decreases the temperature of the gel-to-liquid-crystalline phase transition and increases the permeability of the

lipid bilayer to nonelectrolytes. These results have been of major importance in explaining the effects of membrane lipid acyl chain composition on such properties as membrane permeability and 'fluidity'.

One major limitation of the studies just discussed is that they have used only PC's with n-acyl chains of even carbon numbers. While such acyl chains are the most common in higher animals, a variety of other chain structures, including odd-chain n-alkyl, isobranched, anteisobranched and alicyclic (especially cyclopropyl) species, occur in nature as major components of the total membrane fatty acyl groups in a variety of organisms, especially in plants and microorganisms (Gunstone, 1967; Christie, 1973; Kaneda, 1977). As well, these and other acyl chains (including trans-unsaturated species) are frequently incorporated into the membrane lipids of various organisms or cultured cells using in vivo lipid manipulation techniques (McElhaney and Tourtellotte, 1969; Cronan and Gellman, 1975; Horwitz et al., 1978). To date, the physical properties of pure lipids containing non-n-alkyl or -alkenyl chains have not been explored in any systematic manner. This situation is at least partly attributable to the high cost of many branched-chain and cyclopropane fatty acids, as few biochemical workers would wish either to invest several thousand dollars in the synthesis of a few novel PC's or to undertake the rather specialized syntheses required to produce the needed fatty acid species. Over the past two years, I have developed new and simpler syntheses of iso- and anteisobranched fatty acids (Silvius and McElhaney, 1979a,d) which allow them to be prepared in gram quantities at modest cost (including the natural l-anteiso acid stereoisomers which are not commercially available). I have used these methods, plus established procedures for the synthesis of cyclopropane fatty acids (Christie

et al., 1968; Gunstone and Perera, 1973) and a new synthesis of PC's (Patel et al., 1979), to prepare a number of new diacyl PC's. I have also used differential thermal analysis (DTA) to study the thermotropic phase transitions of these new PC species.

Synthetic Pathways and Procedures

The basic reaction in the synthesis of branched-chain fatty acids is the coupling of an acetylenic molecule (as the lithio or sodio anion) with a molecule containing a bromo group, using liquid ammonia (b.p. -35°) as the solvent:

$$X\text{-}(CH_2)_n C{\equiv}CH \;+\; Br(CH_2)_m\text{-}Y \xrightarrow[NH_{3(\ell)}]{LiNH_2 \text{ or } NaNH_2} X\text{-}(CH_2)_n C{\equiv}C(CH_2)_m\text{-}Y$$

Normally one of the groups X- or Y- was a carboxyl group (converted to a nonreactive carboxylate group by reaction with an extra equivalent of the alkali amide) while the second was a branched alkyl moiety (either sec-butyl or isopropyl for anteiso- or isobranched acids, respectively). For the synthesis of the longer-chain isobranched acids and all of the anteisobranched acids, group X- was the carboxyl moiety, while for the shorter iso- acids (C_{12}-C_{14}), it was the isopropyl moiety (commercial isoheptyne was used as the acetylene in these three cases). The ω-acetylenic fatty acids were synthesized by condensation of ω-bromo acids with lithium acetylide in liquid ammonia (Ames and Covell, 1963) or by bromination/sodamide dehydrobromination of ω-olefinic acids (Khan et al., 1963). The alkyl bromides used were commercial isopentyl bromide (for isobranched acids) and ℓ- and dl-1-bromo-3-methylpentane (for ℓ- and dl-anteisobranched acids). The latter species were synthesized from ℓ- or dl-2-methyl-1-butanol by the following series of reactions (Silvius

and McElhaney, 1979d):

$$\text{R-OH} \xrightarrow[\text{TEA}]{\text{MsCl}} \text{R-OMs} \xrightarrow[\text{aq. EtOH, }\Delta]{\text{KCN}} \text{R-CN} \xrightarrow[\Delta]{\text{aq. KOH}} \text{R-COOH}$$

$$\xrightarrow[H_2SO_4]{\text{MeOH}} \text{RCOOMe} \xrightarrow[Et_2O]{LiAlH_4} RCH_2OH \xrightarrow[\text{TEA}]{\text{MsCl}} \xrightarrow[Et_2O]{MgBr_2} RCH_2Br$$

The overall yield was ∿30% of pure bromide after distillation (b.p. 41°/14 mm). It is not necessary to synthesize dl-1-bromo-3-methylpentane by this full procedure, as dl-3-methyl-1-pentanol is commercially available. In the present study, I used the racemic 2-methyl-1-butanol as starting material in the above synthesis to test the efficiency of the procedure before using the rather costly stereospecific alcohol as the starting compound.

Once the coupling of the acetylenic and bromo-substituted components was accomplished (usually in yields of 30-80%, excepting only the C_{19} anteiso- acids), the acidic products were isolated by selective extraction into aqueous alkali and re-extraction into chloroform or hexane after acidification of the aqueous phase. There were no detectable acidic side-products in the crude reaction products when the lithamide/liquid ammonia coupling system was used, while the butyllithium-hexamethylphosphoramide coupling procedure (Gilman and Holland, 1974) invariably gave substantial amounts of an ill-defined reddish material which was difficult to wholly remove from the products. The residual acidic starting material was removed by differential solvent partition (between hexane and methanol/water), to remove short-chain ω-bromo acids, or by treatment of the methyl esters with silver nitrate, either in ethanolic

solution or adsorbed to silicic acid (Christie, 1973), to precipitate ω-acetylenic species as highly insoluble silver salts. Catalytic hydrogenation of the branched-chain acetylenic acids or esters thus purified gave the saturated species in quantitative yield. The details of these synthetic procedures are discussed elsewhere (Silvius and McElhaney, 1979a,d).

Cyclopropane fatty acids were synthesized by reacting an olefinic ester with methylene iodide and a zinc-copper couple to give the corresponding cyclopropane derivative (Christie et al., 1968; Gunstone and Perera, 1973). The purified cyclopropane and branched-chain acids synthesized by me, plus several commercially available odd-chain and unsaturated fatty acids, were used to synthesize diacyl PC's. All fatty acids used were of 99+% purity by gas-liquid chromatography of the methyl esters and had melting points (see Table 10) in good agreement with literature values where the latter were available (Abrahamsson et al., 1963; Hofman et al., 1954, 1957).

The procedure used for the synthesis of PC's involved the coupling of a fatty acid anhydride (produced by treating the free fatty acid with dicyclohexylcarbodiimide in dry carbon tetrachloride (Selinger and Lapidot, 1966)) with the anhydrous cadmium adduct of glycerophosphorylcholine in dry benzene/dimethylsulfoxide, using 4-pyrrolidinylpyridine as catalyst (Patel and Sparrow, 1979; Patel et al., 1979). The products were isolated and purified by column and thin-layer chromatography (TLC) to give pure PC's; the acyl chain purity was determined by gas-liquid chromatography of the derived methyl esters and the absence of other lipids (especially of lyso-PC) was established by analytical TLC. Only PC's of >99% estimated purity by both of these criteria were used for DTA analysis of their thermotropic behavior.

Experimental Results and Implications

General Thermotropic Behavior of Various Phosphatidycholine Classes

- The different classes of PC's (odd-chain n-acyl, isobranched acyl, etc.) show rather different thermotropic behavior when dispersed in excess (50%, w/w) water. Representative thermograms for a set of PC's of different acyl chain structures are shown in Figure 12. All of the thermograms are dominated by a major sharp endothermic peak, which I assign to a gel-to-liquid-crystalline lamellar phase transition for two main reasons. First, a major sharp endotherm is the most consistent feature of the thermograms observed for various members of each class of PC's, and such an endotherm has been definitely assigned to the gel-to-liquid-crystalline phase transition for various n-acyl PC's of even carbon number (Melchior and Steim, 1976). If we consider the fact that the saturated n-acyl PC's and the cis-unsaturated acyl PC's exhibit essentially similar gel-to-liquid-crystalline phase transitions, it is evident that such transitions are not limited to PC's of a single type of acyl chain structure. It is thus reasonable to expect that PC's whose acyl chains are singly methyl-branched or contain a cyclopropane ring instead of a double bond might exhibit similar phase transitions. Secondly, the temperatures of the major transition endotherms for various PC's made from Class I fatty acids (see Chapter 3) correlate very well with the midpoint temperatures of the membrane lipid phase transitions in membranes of Acholeplasma laidlawii B whose lipids are made homogeneous in the same fatty acyl species, regardless of the detailed structure of the fatty acyl chains. Experiments using fatty acids of various structures to grow A. laidlawii B have indicated that the membrane lipid transition is a gel-to-liquid-crystalline transition (Engelman, 1971) and has the same effects

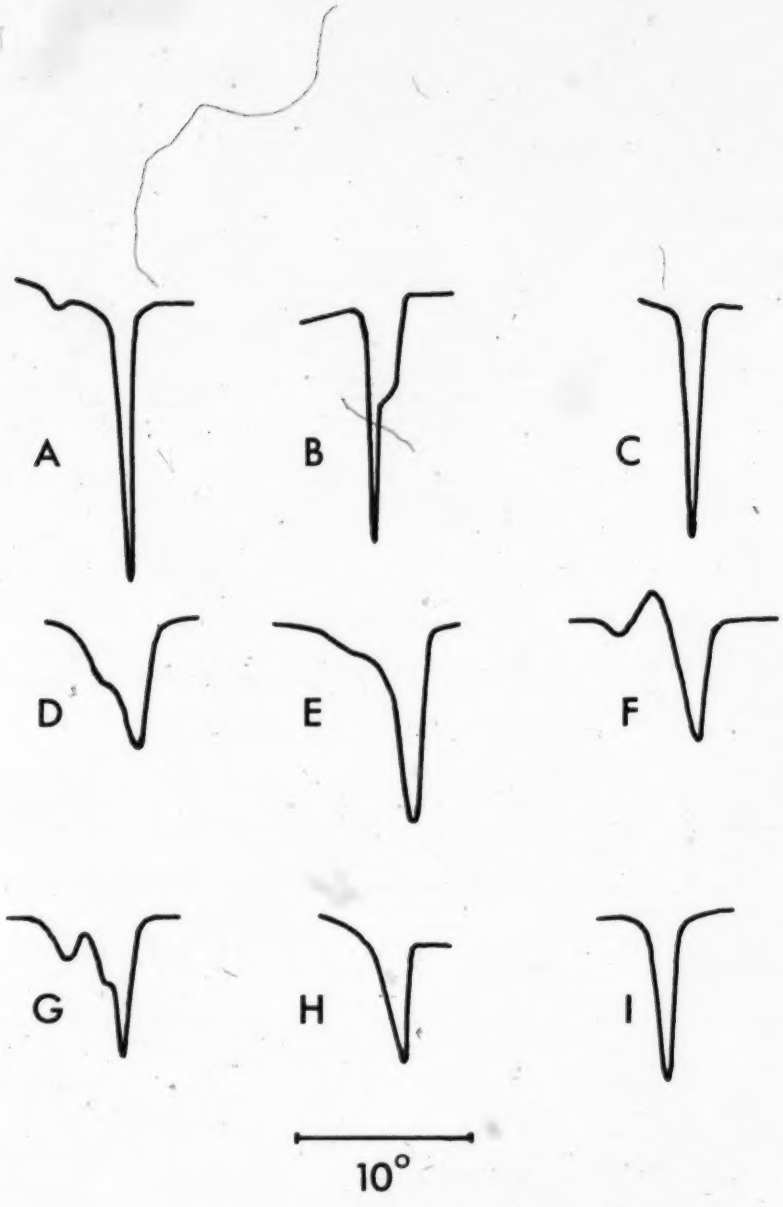


Figure 12. Representative differential thermograms illustrating the thermotropic behavior of various types of hydrated diisoacyl phosphatidylcholines (PC's). The samples shown, and the types of PC's they exemplify, are: A, di-16:0 (longer-chain n-acyl); B, di-13:0 (shorter-chain n-acyl); C, di-18:1tΔ^{11} (monounsaturated acyl); D, di-dl 19:cp,cΔ^{9} (cis-cyclopropane acyl); E, di-dl 19:cp,tΔ^{9} (trans-cyclopropane acyl); F, di-13:0i (shorter-chain isoacyl); G, di-dl 17:0ai (l or dl anteisoheptadecanoyl); H, di-dl 16:0ai (anteisoacyl, save 17:0ai); and G, di-16:0i (longer-chain isoacyl) PC's. The temperature scale is shown by the bar below the thermograms, but T_c values are not given here (see Table 10).

on membrane functional properties regardless of the acyl chain structure (McElhaney, 1974a,b), as we shall see in Chapter 7 of this work as well. Therefore, it seems reasonable to identify the major endothermic transitions of the various PC's with an order-disorder transition of the lipid acyl chains which is basically similar, if not identical, to the gel-to-liquid-crystalline phase transitions observed for pure n-acyl PC's.

The structural bases of the less energetic endothermic transitions observed on heating many of the diacyl PC's studied are less readily explained than is that of the major transition. In two cases, we may advance tentative explanations for these minor transitions as well. In the case of the odd-chain saturated n-acyl PC's, the weakly endothermic transition preceding the major one has been previously observed for certain even-chain n-acyl PC's and has been interpreted as a rearrangement of the lipid acyl chains and/or head-groups which maintains the acyl chains in a quasicrystalline array, much as they are in the gel state (Rand et al., 1975; Janiak et al., 1976). In the case of the cyclopropane acyl PC's, the low-temperature shoulders on the main endotherms are found to decrease in size as the samples are progressively more slowly cooled through their phase transitions prior to recording their heating endotherms. The cyclopropane acids used in this study are actually racemic mixtures (the cyclopropane ring is optically active), and the PC's derived from them are therefore mixtures of four diastereomers; rapid cooling of such mixtures could hinder the proper 'sorting out' of the lipid molecules to form an optimal packing arrangement of the acyl chains in the gel phase. This effect would explain the existence of a low-temperature shoulder (representing the transition of imperfectly ordered regions) on the main cyclopropane PC transition endotherm, and also the gradual disappearance of

this shoulder as the sample is more slowly cooled through the transition. The problem of explaining the minor endothermic transitions of the branched-chain PC's, which occur at temperatures below the main transition temperatures, and the high-temperature shoulders on the main transition peak for ditridecanoyl (13:0) (and dilauroyl, 12:0 (Mabrey and Sturtevant, 1976)) PC's remains a vexing one. It may be that the minor transitions for the branched-chain PC's represent acyl chain and/or headgroup rearrangements within a well-ordered lamellar phase, as the pretransitions do for the saturated n-acyl PC's. In view of the well-documented polymorphism of free fatty acids and other paraffinic compounds in crystalline phases (Malkin, 1952; Chapman, 1965), this proposal is quite reasonable, if highly speculative.

Effect of Acyl Chain Length on T_c Values for Different Phosphatidylcholine Classes - In this section, I shall consider the acyl chain length dependence of the gel-to-liquid-crystalline phase transition temperature, T_c, for PC's in the homologous series studied (i.e., the saturated n-acyl, isoacyl and anteisoacyl PC's). A simple-minded approach to the expected melting behavior of a homologous series of paraffinic compounds suggests that the temperature, T_t, at which any particular phase transition occurs could be calculated as follows:

$$T_t = \frac{\Delta H_{eg} + n\Delta H_m}{\Delta S_{eg} + n\Delta S_m} \qquad [5\text{-}1]$$

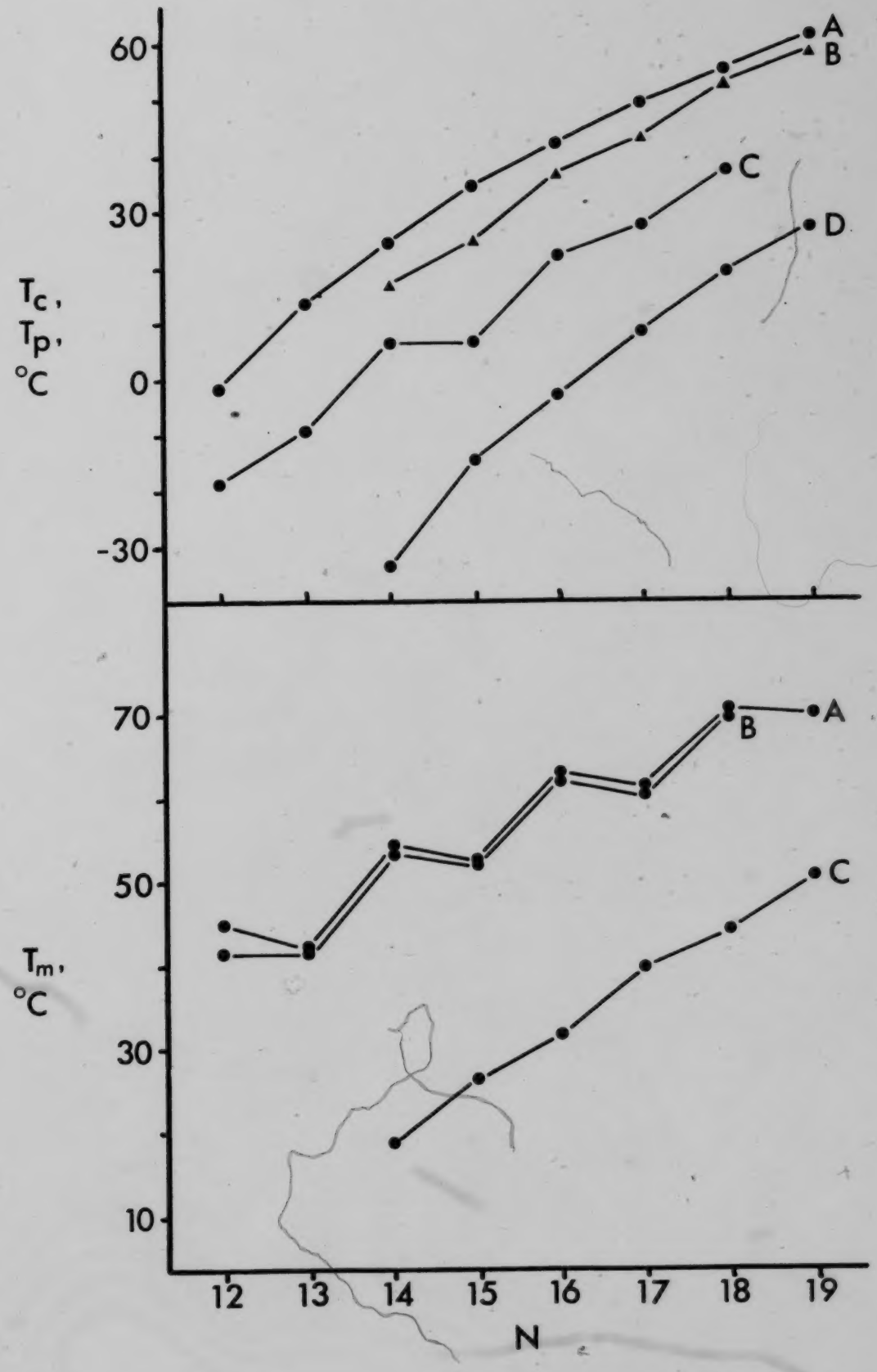
where ΔH_{eg} and ΔS_{eg} represent the (constant) contributions of the endgroups to the transition enthalpy and entropy, respectively, and ΔH_m and ΔS_m represent the (constant) enthalpic and entropic contributions of each of the n methylene groups of the hydrocarbon chain. This result is

reasonable only if the molecular crystal packing arrangement is the same for all of the homologs tested, and only if the hydrocarbon chains are sufficiently long that further addition of methylene groups adds only methylene-methylene interaction terms to the transition free energy. In fact, a variety of homologous series of paraffinic compounds do show phase transition temperatures in reasonable agreement with the predictions of this simple model (Malkin, 1952). However, a more common observation is that the T_t's of the homologs of even chain length fit along one smooth curve, while the T_t's of the odd-chain homologs fit along a different curve. This phenomenon, called 'alternation', stems from some rather elementary aspects of molecular packing. Quite simply stated, if a paraffinic compound of n carbons adopts a certain packing arrangement in a well-ordered (usually crystalline) phase, so can homologs of (n-2), (n+2), etc. carbons. However, it is not possible for a homologous compound of (n-1) or (n+1) carbons to adopt a packing arrangement that is exactly comparable in both the lateral polymethylene chain packing and the arrangements of the end-groups, unless the long polymethylene chain axis and the planes of the molecular end-groups are mutually perpendicular (for a fuller discussion, see Malkin, 1952). If the polymethylene chain axis and the end-group planes are not perpendicular, the T_t's of the odd- and of the even-carbon-numbered species will be described by two distinct forms of equation [5-1], and alternation of T_t values will result. The magnitude and direction of the alternation cannot be predicted *a priori*. However, alternation is generally clearly evident in plots of transition temperatures *vs.* carbon number for the phase transitions of a variety of paraffinic compounds whose polymethylene chains are not strictly perpendicular to the plane of the molecular end-groups in at least one well-ordered phase

involved in the phase transition of interest (Malkin, 1952; Gunstone, 1967).

With these considerations in mind, we turn our attention to the data of Figure 13, where the fatty acid melting points (T_m) and the PC main transition temperatures (T_c) are plotted vs. acyl chain length for all of the saturated normal, isobranched and dl-anteisobranched fatty acids and PC's studied in this work. The pretransition temperatures (T_p) are also given in the case of the saturated n-acyl PC's. In the fatty acid series examined, the normal and isobranched species show strong alternation of T_m values, while the racemic anteisobranched acids show a weak alternation for longer chain lengths (16-19 carbons) and little if any alternation for shorter chain lengths. The former observation suggests that the straight-chain and isobranched saturated fatty acid chains are tilted in the crystalline phase, i.e., that their polymethylene backbones are not strictly parallel to the vector normal to the planes of their end-groups. By contrast, the very weak T_m alternation seen for the racemic anteisobranched fatty acids suggests that these species either crystallize with their polymethylene chains very nearly perpendicular to the planes of their end-groups (which is definitely not the case for the ℓ-isomers at least (Abrahamsson et al., 1963)) or fail to form a well-ordered crystalline phase in which the orientations of the methyl-terminal portions of the acyl chains are well-defined. I favor the latter explanation, for the ℓ-anteisobranched fatty acids melt at temperatures very close to the T_m's of the corresponding racemic mixtures, suggesting that the acyl chain packing arrangements are very similar. This similar packing would seem to be possible only if the methyl-terminal portions of the acyl chains are rather disordered and loosely packed in the crystal.

Figure 13. Dependence of fatty acid melting points, T_m, and phosphatidylcholine (PC) gel-to-liquid-crystalline phase transition and pretransition temperatures, T_c and T_p, respectively, on fatty acyl chain length. In the top graph, the T_c values are given for: A, the saturated n-acyl PC's; C, the isoacyl PC's; and D, the dl-anteisoacyl PC's, and T_p values are given for: B, the n-acyl PC's. In the lower graph, the free fatty acid melting points are given for: A, the straight-chain saturated fatty acids; B, the isobranched fatty acids; and C, the dl-anteisobranched fatty acids.


60
30
0
-30
T_c, T_p, °C
A
B
C
D
70
50
30
10
T_m, °C
A
B
C
12
13
14
15
16
17
18
19
N

If we turn to an examination of the T_p and T_c values for the saturated n-acyl and isoacyl PC's, we find that the T_c values for the isoacyl PC's clearly alternate, as do the T_p values for the n-acyl PC's, while the T_c values for these latter species fall along one smooth curve (Figure 13). By reasoning similar to that used in the foregoing discussion of fatty acid crystal structures and melting points, I come to the conclusion that at least one well-ordered phase involved in the isoacyl PC gel-to-liquid-crystalline phase transition exhibits a net tilt of the acyl chains, as does at least one of the phases involved in the n-acyl PC pretransition. By contrast, any well-ordered phase(s) involved in the n-acyl PC gel-to-liquid-crystalline phase transition would appear to exhibit no net chain tilt. Therefore, in the case of the saturated n-acyl PC's, I conclude that the phase existing below T_p exhibits a finite acyl chain tilt, while that existing above T_p but below T_c does not. The liquid-crystalline phase of saturated n-acyl PC's does not have an orderly arrangement of the acyl chains (Tardieu et al., 1973), and therefore I cannot make any conclusions regarding the presence of tilting of the acyl chains in this phase on the basis of T_c data. Reasoning entirely similar to that just described indicates that the isoacyl PC hydrocarbon chains are tilted in the gel state. My conclusions regarding the phase behavior of the saturated n-acyl PC's agree with the conclusions of certain other workers using X-ray diffraction (Rand et al., 1975; Brady and Fein, 1977), while my findings are rather more difficult to explain by the proposal of certain other X-ray diffraction workers (Janiak et al., 1976; Larsson, 1977) that the acyl chains in the phase existing between T_p and T_c remain tilted. I cannot absolutely rule out the possibility that a disordering of the acyl chains sufficient to destroy all order in

their methyl-terminal regions occurs on passage of the n-acyl PC's through the pretransition. Brady and Fein (1977) have in fact interpreted their X-ray results as representing a ~20% decrease in acyl chain ordering at T_p. However, I consider the above possibility unlikely, as Raman spectroscopic results (Gaber et al., 1978) indicate that the decrease in chain ordering at T_p is slight (roughly 1 gauche conformation per chain), which would seem to be too small to abolish all ordering of the methyl-terminal portions of the PC acyl chains.

The variation of the gel-to-liquid-crystalline phase transition temperature with acyl chain length for the anteisoacyl PC's does not clearly exhibit alternation (Figure 13), nor do the T_c values all fit on a single smooth curve of T_c vs. carbon number as do those of the n-acyl PC's. I suggest that the methyl-terminal portions of the acyl chains of anteisoacyl PC's are quite disorganized even in the gel state, and that this structural feature is responsible for the rather ill-defined (neither smoothly curving nor strictly alternating) relationship of T_c to the acyl chain length. This proposal would explain why the l- and dl-anteisoacyl PC's of a given chain length (which differ in their structures in the methyl-terminal portions of their acyl chains) always have very similar T_c values, for if the structure of the methyl-terminal region of the PC bilayer in the gel state is ill-defined, modest structural differences in the acyl chains in this region will not greatly affect the gel-to-liquid-crystalline transition temperature. The above proposal would also explain one other result that I find quite noteworthy, namely the fact that the heating endotherms for samples of hydrated anteisoacyl PC's are generally considerably smaller than those for samples of n- or isoacyl PC's of the same chain length. While results from DTA are difficult to

precisely calibrate in terms of heats of transition, the size of the DTA endotherm is roughly proportional to the heat of transition for a group of samples of constant thermal conductivity (which a set of different PC's dispersed in an excess of water, a good thermal conductor, should be). Therefore, the heats of transition for the anteisoacyl PC's appear to be smaller than are those of the other classes of PC's studied, especially for the shorter chain lengths (14 and 15 carbons, with no transition detectable at all for the 13-carbon anteisoacyl PC). This result would be expected if the anteisoacyl PC's were already somewhat disordered below their phase transition, for then the increase in disorder (and hence in enthalpy) which would result when the lipids entered the liquid-crystalline state would be less than that observed for a PC which had well-ordered acyl chains in the gel state.

Effects of Fatty Acyl Chain Structure on Phosphatidylcholine Phase Transition Temperatures - In Table 10, I have listed all of the T_c values for the diacyl PC's studied here, along with the melting points of the fatty acids used in their preparation. As the dependence of T_c on the PC acyl chain length has been discussed in some detail in the last section, I wish to turn here to an examination of the effect of fatty acyl chain structures on T_c at a fixed acyl chain length. If we consider the PC's whose acyl chains have linear hydrocarbon 'backbones' of fifteen carbons (dipalmitoyl (16:0), diisoheptadecanoyl, dipalmitoleoyl (16:1cΔ^9), and di-dl-cis-9,10-methylenehexadecanoyl (17cp,cΔ^9) are some examples), we find the order of T_c values to be as follows: n-acyl (41.5°C) > isoacyl (27°) > dl-anteisoacyl (8.0°) ≈ ℓ-anteisoacyl (7.6°) > trans-cyclopropane (-0.3°) > trans-monounsaturated (-4.0°) > cis-cyclopropane (-19.9°) > cis-monounsaturated (-35.5°). Quite similar orderings are found for other

Table 10

Melting Points (T_m) of Various Fatty Acids and Gel-to-Liquid-Crystalline Phase Transition Temperatures (T_c) of their Fully Hydrated Phosphatidylcholine Derivatives

Fatty Acid	T_m of Acid (°C)	T_c of PC (°C)	Fatty Acid	T_m of Acid (°C)	T_c of PC (°C)
12:0	44.8	-1.8 [a]	dl 18:0ai	44.0	18.7
13:0	41.8	13.5	dl 19:0ai	50.2	26.8
14:0	54.4	24.0			
15:0	52.5	34.2	ℓ 15:0ai	24.0	-16.5
16:0	62.9	41.5	ℓ 17:0ai	36.9	7.6
17:0	61.3	48.8	ℓ 19:0ai	48.2	26.4
18:0	70.1	54.8			
19:0	69.4	60.9	16:1cΔ^9	3.1	-35.5
			16:1tΔ^9	33.2	-4.0
12:0i	41.4	-18.5	18:1cΔ^9	10.5	-15.8
13:0i	41.3	-9.5	18:1cΔ^{11}	13.0	-19.5
14:0i	53.4	6.5	18:1tΔ^9	45.0	12.9
15:0i	51.5	6.5	18:1tΔ^{11}	44.0	13.2
16:0i	62.2	22			
17:0i	60.0	27	dl 17:cp,cΔ^9	18.6	-19.9
18:0i	69.5	36.5	dl 17:cp,tΔ^9	26.1	-0.3
			dl 19:cp,cΔ^9	39.3	-0.5
dl 14:0ai	18.5	-33.7	dl 19:cp,cΔ^{11}	31.8	-3.5
dl 15:0ai	26.3	-14.8	dl 19:cp,tΔ^9	33.9	16.3
dl 16:0ai	31.9	-3.0	dl 19:cp,tΔ^{11}	36.5	14.0
dl 17:0ai	39.6	8.0			

[a] Value reported by Mabrey and Sturtevant (1976).

chain lengths, although some structures may not be represented in any one series. We can draw the following general conclusions about the effects of acyl chain structure on phosphatidylcholine T_c's:

(1) Distortions of the acyl chain structure from a linear all-trans polymethylene arrangement generally decrease T_c. Such distortions have more effect when present in the middle of the acyl chain than when present near the methyl terminus. It is worth noting in this respect that di-dl-10-methyloctadecanoyl PC has been recently found to have a T_c below 0°C, which is considerably lower than the T_c of 26.8° for di-dl-16-methyloctadecanoyl PC studied here, and that Barton and Gunstone (1975) have shown that di-cis-16-octadecenoyl PC has a T_c of 35°C, while I found di-cis-9-octadecenoyl PC to have a T_c of -15.7°C.

(2) A trans-double bond lowers T_c less than does a cis-double bond at the same position in the acyl chain. Replacement of the double bond by a cyclopropane ring of like configuration raises T_c significantly for cis-unsaturated acyl PC's but only slightly for trans-unsaturated acyl PC's.

(3) Both iso- and anteisobranched acyl chains depress the T_c value for a PC significantly below that of the corresponding n-acyl species. These naturally occurring branched-chain acids thus serve as membrane lipid 'fluidizers' when they replace saturated n-acyl chains in the lipids. The anteisobranched acids are more effective in this respect than are the isobranched acids, but they are still less effective than naturally occurring unsaturated fatty acids.

(4) The PC acyl chain structure can affect the chain length dependence of the PC's major transition temperature. For example, an increase in the linear polymethylene 'backbone' length of the PC fatty acyl chains

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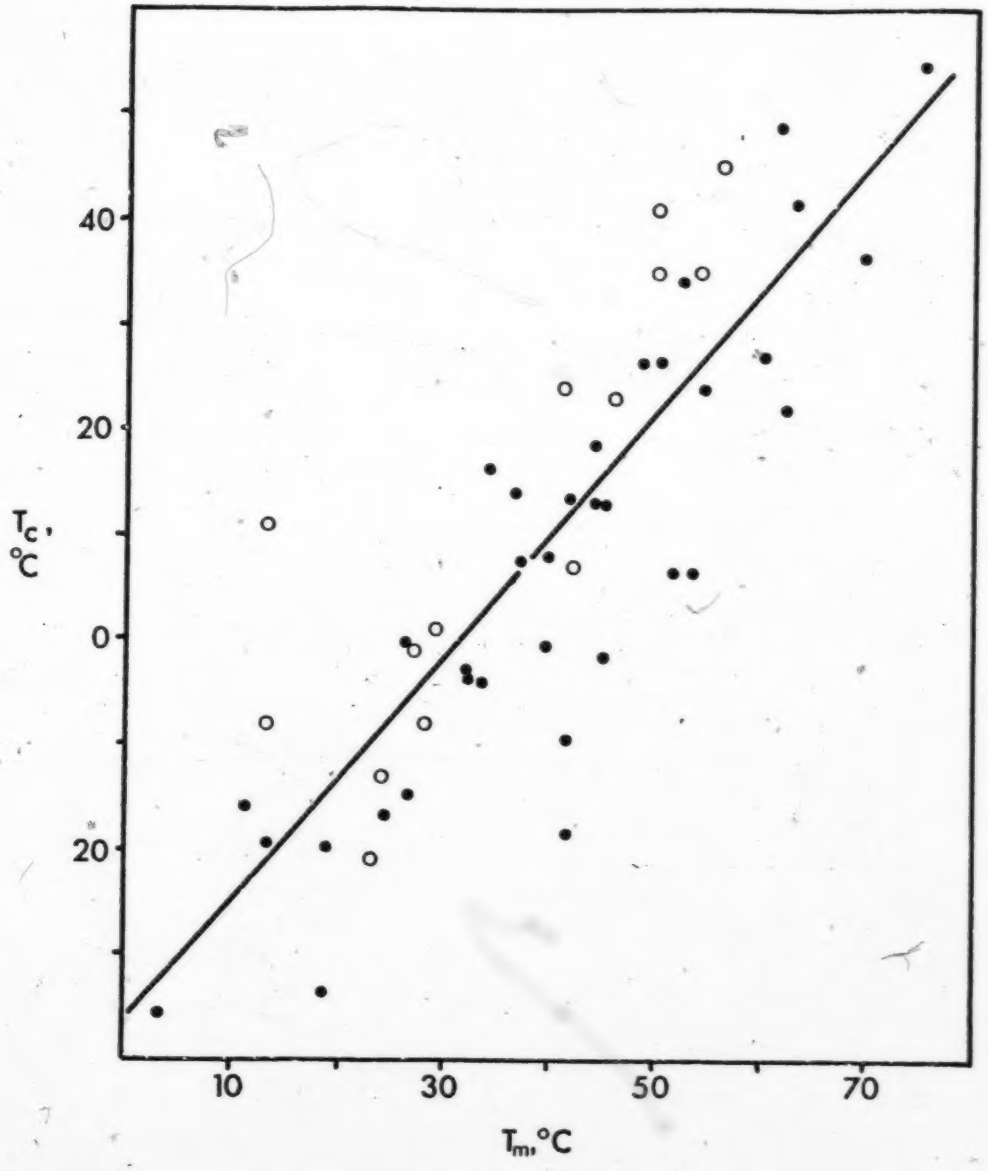

Figure 14. Correlation of the melting points, T_m, of various free fatty acids with the gel-to-liquid-crystalline phase transition temperatures, T_c, of the corresponding hydrated diacyl phosphatidylcholines (PC's). The filled circles represent data derived from the studies reported here, while the data represented by the open circles are taken from Barton and Gunstone (1975). The measured correlation coefficient is 0.84.

calculated for a plot of fatty acid T_m's *vs.* the transition midpoint temperatures (T_{AL}) of total *A. laidlawii* B membrane lipids made homogeneous in the same fatty acyl species (see Table 9, Chapter 4) is 0.75, indicating that fatty acid T_m values are even less useful as predictors of T_{AL} than they are as predictors of T_c for pure diacyl PC's. By contrast, the correlation coefficient is calculated to be 0.944 for a plot of the T_{AL} values for fatty acid-homogeneous *A. laidlawii* B lipids *vs.* the T_c values for the corresponding diacyl PC's (Figure 15). Therefore, as one might expect from considering the nature of the phase transitions in the three lipid systems considered above, the transition behavior of the two hydrated diacyl glycerolipid systems is much more regularly related than the melting behavior of the anhydrous fatty acid system is with either of the former systems. I conclude, therefore, that the phosphatidylcholine T_c values reported here form a far more reliable basis for the evaluation and prediction of the effect of fatty acyl composition on the transition behavior of hydrated polar glycerolipids than do the anhydrous fatty acid melting points, which have often been used for this purpose in the past (for example, see Kaneda, 1977). I would also note, however, that since the slope of the T_c - T_{AL} relationship is significantly different from unity (Figure 15), an *identity* of transition temperatures in various hydrated polar glycerolipid systems of like fatty acid composition cannot be assumed, although it would seem that a *systematic relationship* of the transition temperatures in the two systems under conditions of varying fatty acid composition can be assumed. This conclusion is supported by the results of studies of two classes of synthetic lipids, the diacyl phosphatidylcholines and the diacyl phosphatidylethanolamines (PE's) (Van Dijck *et al.*, 1976), where the relatively

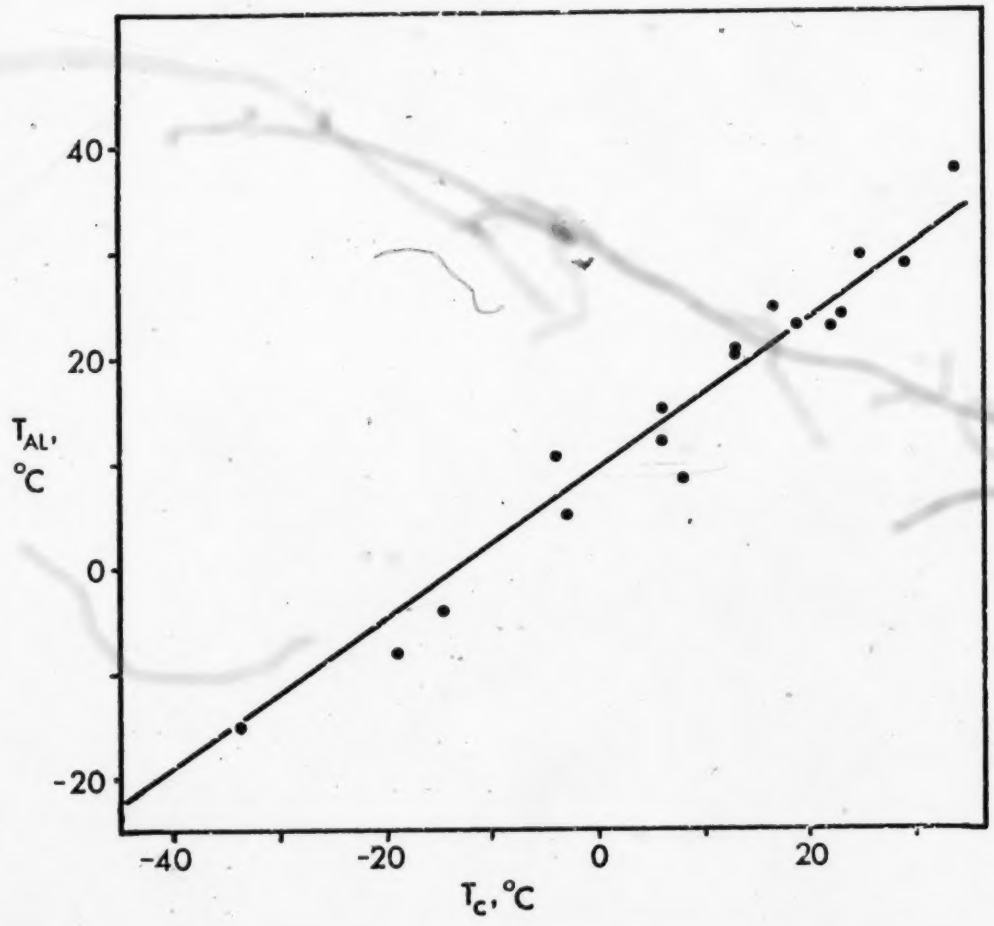

Figure 15. Correlation of the gel-to-liquid-crystalline phase transition temperatures, T_c, for various hydrated diacyl phosphatidylcholines with the transition midpoints, T_{AL}, for hydrated membrane lipids of A. laidlawii B made homogeneous in the same fatty acids. The measured correlation coefficient is 0.944, and the coefficient of regression of T_{AL} or T_c is 0.71.

greater effect of changes in fatty acid structure on the observed T_c values of the latter lipids may be a consequence of the smaller head-group size of the PE's, which may allow their fatty acyl chains to interact more strongly than those of the PC's.

Conclusions

The studies described here were intended to provide basic data regarding the effect of certain branched and alicyclic fatty acyl chains on lipid physical properties in a chemically well-defined system. I have paid most attention to the major transition endotherms determined for the various diacyl PC's studied by DTA, for while the minor transitions may reveal certain important aspects of the lipid acyl chain arrangements in the gel state, such transitions in the case of the saturated n-acyl PC's are characteristic of the pure PC's and are abolished in the presence of small amounts of other lipids or even of other PC species (Mabrey and Sturtevant, 1976). In at least one case, however, the effect of lipid acyl chain structure on the arrangement of the lipids in the gel state may be functionally significant: Verkleij and Ververgaert (1975) have reported that incorporation of substantial amounts of isobranched fatty acids into the lipids of A. laidlawii B or Bacillus subtilis inhibits the lateral aggregation of intramembrane particles below the lipid phase transition temperature that is normally seen by freeze-fracture electron microscopy. Studies using X-ray or electron diffraction could furnish more information regarding the arrangement of the acyl chains in the gel phase for a variety of PC's of different acyl chain structure (Hui, 1976; Brady and Fein, 1977). However, since such studies have given somewhat ambiguous results even for saturated n-acyl

PC's, the potential of this approach when applied to other PC's may prove to be frustratingly limited.

The effects of acyl chain structure on the transition temperature of a pure PC can be at least qualitatively understood in terms of the energies of lateral acyl chain association, as Salem (1962) proposed some time ago. However, fatty acid melting points and the corresponding phosphatidylcholine T_c values show only a fair correlation, and phosphatidylcholine T_c's and fatty acid-homogeneous A. laidlawii B membrane lipid transition midpoints, T_{AL}, have well-correlated but generally nonidentical values (see Figure 15). It would seem, therefore, that the magnitude of the effect of changing one type of acyl chain for another on the phase transition temperature of a given lipid system can vary considerably depending on the nature and structure of the system. I would suggest that the anhydrous fatty acids provide a rather poor basis for assessing the effects of fatty acyl chain structure on the physical properties of a given lipid system, and that, wherever possible, the diacyl PC's derived from a novel fatty acid of interest should be synthesized and characterized in order to obtain a realistic assessment of the effect of that fatty acid on a membrane lipid system. Even in this case, however, differences in lipid head-groups from system to system may significantly modify the detailed nature of the effect of that acyl species on lipid properties.

CHAPTER 6

CHARACTERIZATION OF THE MEMBRANE ADENOSINETRIPHOSPHATASE OF *A. laidlawii* B

Background

In the next two chapters, I shall be developing my experimental and theoretical analysis of the effects of lipid properties on the activity of a membrane-bound enzyme, the Mg^{2+}-dependent adenosinetriphosphatase (ATPase), in *A. laidlawii* B. To place my kinetic studies on a secure footing, I began by determining the dependence of the enzyme activity on substrate and inhibitor concentrations and on the levels of various ions. The results I obtained suggested a possible physiological role for this enzyme as an ion-extruding cellular osmoregulator, and experiments designed to test this hypothesis were designed and carried out. These kinetic and functional studies of the ATPase are described in this chapter.

Adenosine 5'-triphosphate β,γ-phosphohydrolases (ATPases) are ubiquitous in living systems and generally function to convert the chemical energy of the β,γ-phosphoanhydride bond of ATP into various other forms of energy. In muscle, for example, the ATPase of myosin produces mechanical energy, while in the firefly, the luciferin/luciferase system (which includes an ATPase activity) produces light energy, and in the electric organ of eels and rays, the (Mg^{2+},Na^{+},K^{+})-ATPase produces electrical energy. Membrane-bound ATPases from a wide variety of sources appear to function as ion-transport proteins, converting the ATP chemical-bond energy into the electrochemical potential energy of a transmembrane ionic gradient (Tsuchiya and Rosen, 1975; Ronquist, 1975).

ATPases of this type are very resistant to solubilization by changes in ionic strength or pH and are usually released from the membrane only when it is disaggregated by detergents or solvents, suggesting a strong hydrophobic association with the membrane hydrocarbon core. The most common type of membrane ATPase in bacteria consists of two components, a tightly membrane-bound BF_o subunit embedded in the bilayer core and a BF_1 subunit which is easily solubilized by chelators and which carries the ATPase activity (Harold, 1977). ATPases of the BF_o-F_1 type are usually activated by Mg^{2+} and by Ca^{2+} but not by alkali metal cations and act as primary proton pumps.

A tightly membrane-bound ATPase was discovered by Rottem and Razin (1966) in isolated membranes of A. laidlawii (oral strain), but these authors were unable to demonstrate any monovalent cation stimulation of the ATPase activity or to correlate the activity with the uptake of K^+ by energized cells (Cho and Morowitz, 1969). The physiological function of this enzyme has remained obscure. The ATPase is one of the few tightly membrane-bound enzymes which have been described in A. laidlawii B, and its substrates are water-soluble, a fact that facilitates kinetic analysis in terms of simple models which are not applicable to enzymes whose substrates are membrane-bound or micellar (Gatt and Bartfai, 1977). I have characterized the enzyme in membranes of A. laidlawii B in an effort to elucidate its physiological role and to allow me to collect reliable data on the temperature dependence of its activity (see Chapter 7).

Results and Implications

To avoid incorrect interpretations of my kinetic data, I began

my investigation by determining the conditions under which enzyme activity is optimal. The pH dependence of activity is essentially flat between pH 7.0 and 8.0, and the enzyme was therefore assayed at pH 7.4. Mg^{2+} is essential for activity; a low residual activity observed in Mg^{2+}-free buffers is wholly abolished by including 1 mM EDTA in the assay solution. A Mg^{2+} concentration of 15 mM gives maximal activation of activity throughout the temperature range studied. ADP in the assay medium competitively inhibits the ATPase activity with a K_I of 1.9 mM (determined from a Dixon plot of 1/v vs. [ADP]) and is hydrolyzed at roughly 2% of the rate at which ATP is cleaved. To avoid product inhibition by ADP, I sampled the ATP digestion mixture frequently and adjusted the amount of membrane protein in the mixture to ensure that less than 20% of the ATP was hydrolyzed by the end of the assay period. The activity showed a simple hyperbolic relationship to ATP concentration up to 1 mM ATP, but ATP concentrations above 3 mM increasingly strongly inhibited the enzyme. Therefore, the enzyme V_{max} was determined by measuring the reaction rate (v) at various ATP concentrations up to 1 mM and plotting [ATP]/v vs. [ATP]. This method of data plotting yields $1/V_{max}$ from the slope of the graph and $-K_m$ from the X-intercept, and it gives K_m and V_{max} estimates which are less strongly biased than are those derived from Lineweaver-Burk or Eadie-Hofstee plots (Wilkinson, 1961).

Measurement of the enzyme's K_m and V_{max} as functions of temperature reveals that both parameters increase with increasing temperature from 5° up to at least 40°C regardless of the membrane lipid fatty acid composition. However, the temperature dependence of K_m does not seem to be influenced by membrane fatty acid composition, while, as will be discussed in the next chapter, the temperature dependence of V_{max} is

strongly affected by the fatty acid composition. In Figure 16, the variation of K_m with temperature is shown for the ATPase in membranes enriched with no fatty acid, or with elaidate or linoleate (with lipid gel-to-liquid-crystalline transition midpoint temperatures of 34°, 21° and -18°C, respectively (McElhaney, 1974a)). The data shown in this figure reveal no significant dependence of K_m on membrane fatty acid composition or on the temperature range of the lipid phase transition.

In experiments in which V_{max} and K_m were determined at many temperatures, it was often difficult to ensure optimal assay conditions (protein concentration and sampling times) for all temperatures, as it was necessary to obtain significant (i.e., easily measurable) release of P_i, while avoiding hydrolysis of a significant fraction (>20%) of the ATP, in order to measure a true initial rate during the assay period. This balance of conflicting factors was especially delicate at low ATP concentrations, and in routine experiments using many assay temperatures, ATP concentrations below 200 μM were not normally used. This limitation made the determination of K_m inherently less precise than that of V_{max}, as ATP concentrations far below K_m were not utilized. Nonetheless, the results of a number of studies of the temperature dependence of K_m have supported my above conclusion that the variation of K_m with temperature is not affected by the fatty acid composition, or the temperature of the phase transition, of the membrane lipids. This result indicates that the geometry of the ATP-binding site is not altered by a lipid phase transition, a finding which in turn strongly suggests that the ATPase does not undergo an overall change of conformation within the membrane lipid phase transition range.

The membrane ATPase of A. laidlawii (oral strain) was found by

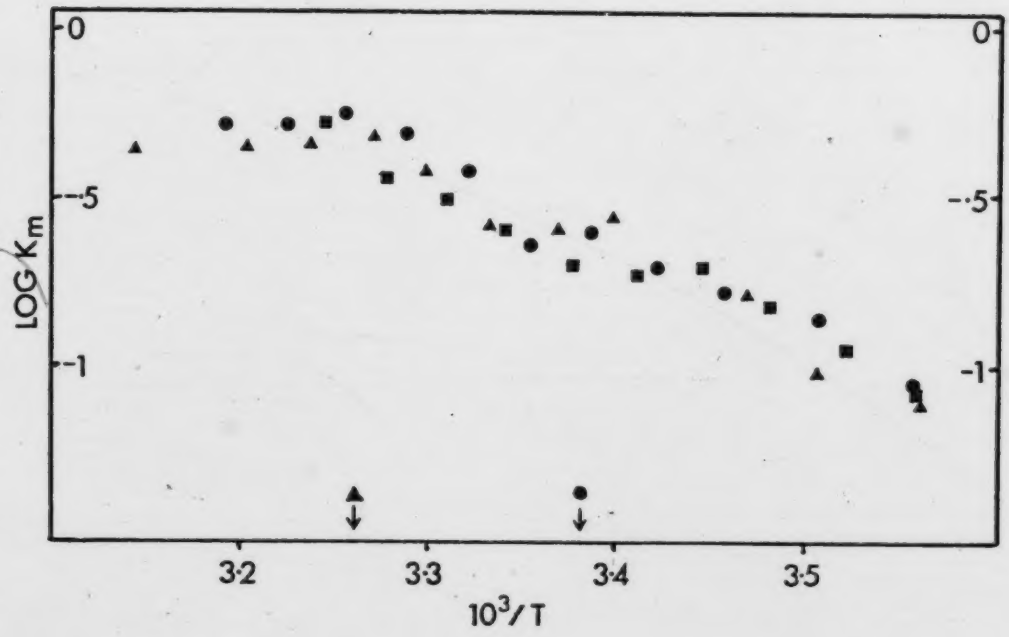

Figure 16. Variation of K_m for ATP (in mM), in the presence of excess Mg^{2+}, with temperature for the (Na^+,Mg^{2+})-ATPase activity in A. laidlawii membranes enriched in linoleic acid (●), elaidic acid (■), or palmitic plus myristic acids (▲). The arrows along the (10^3/T) axis indicate the lipid phase transition midpoints for the latter two membrane preparations, determined by differential thermal analysis (the phase transition in linoleate-enriched membranes lies entirely below 0°C).

Rottem and Razin (1966) to be inhibited by sulfhydryl group reagents such as organomercurials and N-ethylmaleimide (NEM). Bacterial $BF_o \cdot F_1$-type ATPases are extremely sensitive to inhibition by lipophilic carbodiimides. To test the sensitivity of the A. laidlawii B ATPase to such compounds, isolated membranes were incubated with various inhibitors for a fixed time, and the incubation mixture was then diluted at least fifty-fold and assayed for residual ATPase activity. From the dependence of inhibition on the inhibitor concentration, a K_I was calculated for each inhibitor, using a rapid binding - slow inactivation model of enzyme-inhibitor association as described in Appendix 3. As shown in Figure 17, both dicyclohexylcarbodiimide (DCCD) and p-chloromercuribenzenesulfonate (PCMBS) inhibit the enzyme in a manner consistent with the model of Appendix 3, as is evidenced by the linearity of the inhibition plots. The linearity of the inhibition graphs over a wide range of fractional inhibitions of the ATPase activity strongly suggests that a single enzyme species catalyzes all or nearly all of the measured ATP hydrolysis, for a second ATPase activity of any magnitude would cause the graphs to be nonlinear unless it exhibited K_I values which were very similar to those of the first enzyme for both PCMBS and DCCD. I consider this possibility quite unlikely.

From the data of Figure 17, K_I values of 100 μM and 170 μM are found for ATPase inhibition by DCCD and PCMBS, respectively. The K_I value for DCCD is much larger than that found in bacterial $BF_o \cdot F_1$ ATPases, suggesting that the A. laidlawii enzyme is quite distinct from these enzymes. Inhibition of the ATPase by NEM gave a nonlinear graph when plotted in the manner of Figure 17, but the major phase of inhibition gave a K_I of $\sim$ 2.2 mM. The K_m of the ATPase is unaltered when V_{max} is

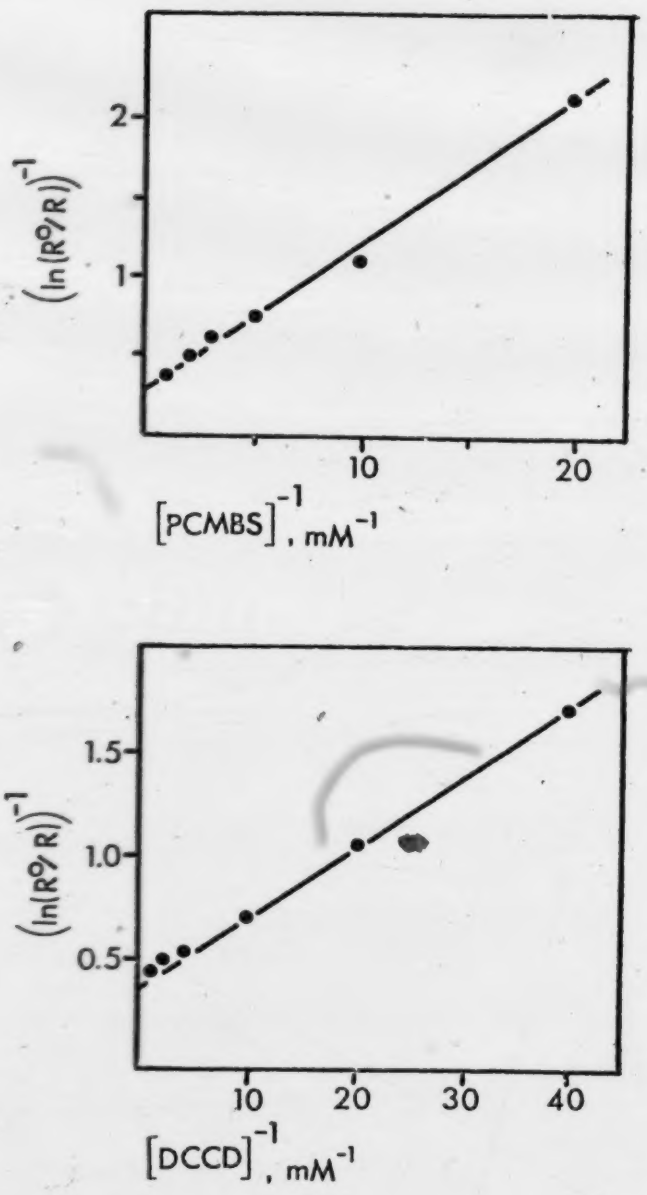

Figure 17. Inhibition of the (Na^+,Mg^{2+})-ATPase in membranes of fatty acid-unsupplemented A. laidlawii B by p-chloromercuribenzenesulfonate (PCMBS) or dicyclohexylcarbodiimide (DCCD). The plots shown follow the analysis described in Appendix 3 and give inhibitor dissociation constants, K_I, of 170 μM for PCMBS and 100 μM for DCCD. Experimental details are given in the text.

reduced by 65% by pretreatment with 10 mM NEM, suggesting that the non-linear plots of NEM inhibition noted above cannot be attributed to the presence of multiple ATPase species in the membrane (unless all of these have a very similar K_m, which is rather unlikely). This conclusion of a unique ATPase in the membrane is consistent with my results with DCCD and PCMBS inhibition.

To demonstrate that the (Mg^{2+},Na^+)-ATPase and the (Mg^{2+})-ATPase activities are catalyzed by the same enzyme, I compared the K_m's and the sensitivities to PCMBS and NEM inhibition of the two activities. The enzyme's K_m was not detectably altered by 300 μM or 10 mM Na^+, while V_{max} increased approximately fourfold at the higher Na^+ concentration, indicating that the Na^+ activation is attributable purely to an enhancement of the enzyme catalytic rate constant(s). Both NEM and PCMBS gave parallel inhibition of the (Mg^{2+},Na^+)- and (Mg^{2+})-ATPase activities: 100 μM PCMBS reduced basal and Na^+-stimulated activities by 61% and 58%, respectively, while 10 mM NEM reduced them by 69% and 68%. Therefore, the (Mg^{2+},Na^+)- and (Mg^{2+})-ATPase activities seem to be catalyzed by a single enzyme species.

The results discussed above indicate that the A. laidlawii B membrane contains a single major ATPase species whose activity is strongly and specifically stimulated by sodium (or lithium) ions. A. laidlawii B derives its metabolic energy wholly from glycolysis (Smith, 1971). and the most likely energy source for ion extrusion would therfore be ATP (or possibly phosphoenolpyruvate, although the latter has not been found to serve as the primary energy source for inorganic ion transport in any system studied to date). It is quite reasonable to postulate, by analogy with a variety of known ion pumps such as the eukaryotic (Na^+,K^+,Mg^{2+})-

ATPase, that the A. laidlawii B membrane (Na^+,Mg^{2+})-ATPase functions in cellular osmoregulation, extruding excess cations which tend to concentrate in a cell to levels above those which would occur at osmotic equilibrium (Christensen, 1975). However, more direct evidence for an osmoregulatory role of the ATPase is clearly desirable. To obtain such evidence, I have studied the effect of inhibitors of glycolysis or ATPase activity on the maintenance of cellular integrity in various media.

The most convenient measure of cellular integrity is the turbidity of cell suspensions, which responds to both cell swelling and lysis (Van Zoelen et al., 1975). Since swelling and lysis are intimately related processes (lacking a cell wall, A. laidlawii swells until it lyses when osmotically stressed), turbidity provides a useful semiquantitative measure of cellular integrity, decreasing steadily as cells swell and lyse. In a typical experiment, freshly harvested cells were washed once in a test buffer whose osmolarity was adjusted to be equivalent to that of a standard buffer (0.154 M NaCl, 40 mM Tris-H^+, 10 mM Tris, pH 7.4). The washed cells were resuspended in the test buffer to a turbidity of 1.0 absorbance units at 450 nm and were incubated at 35°C with glucose or various inhibitors. The absorbance (turbidity) of the sample was monitored for 90 minutes (at which time lysis was maximal in samples treated with high levels of inhibitors), and the sample was then microscopically examined for evidence of lysis. The results of such experiments indicate that in alkali cation buffers, inhibitors of glycolysis or of the membrane ATPase activity promote cell lysis and glucose preserves cellular integrity, while in solutions of impermeant nonelectrolytes or polyvalent ions, these compounds have little effect on cell stability. This phenomenon is illustrated by the data of Table 11, where the turbidities

Table 11

Effects of Glycolytic or ATPase Inhibitors on Maintenance of Cellular Integrity of A. laidlawii B in Various Buffers

Inhibitor	Buffer[a]	$\frac{\text{O.D. (90 min)}}{\text{O.D. (glucose, 90 min)}}$[b]
NEM (10 mM)	NaCl	0.41
	KCl	0.44
	Na_2SO_4	0.71
	Sucrose	1.00
	$MgSO_4$	1.02
	Taurine	1.02
NaF (25 mM)	NaCl	0.38
	KCl	0.38
	Na_2SO_4	0.70
	Sucrose	0.92
	Taurine	1.01
PCMBS (0.5 mM)	NaCl	0.29
	KCl	0.34
	Sucrose	1.00

[a] All buffers contained 50 mM Tris, pH 8.0, plus the indicated solute at an osmolarity equivalent to 150 mM NaCl.

[b] The absorbance at 450 nm of a cell suspension treated with inhibitor has been divided by the absorbance of a suspension of the same initial absorbance but incubated with 0.25% glucose for 90 min.

of cell suspensions incubated for 90 min in various buffers with an inhibitor have been divided by the turbidities of samples incubated in the same buffer with glucose (0.25%) instead of the inhibitor added.

The lysis-promoting effects of NaF, but not those of NEM or PCMBS, are antagonized by glucose addition to NaCl or KCl buffers, suggesting that the sulfhydryl group reagents do not exert their effects by acting directly on the glycolytic pathway. To test the possibility that these agents promote lysis by inhibiting the membrane ATPase *in vivo*, the dosage dependence of cell lysis by these inhibitors in the presence of glucose was determined. The concentrations of PCMBS and of NEM which gave a half-maximal decrease in suspension turbidity after 90 min of incubation, $K_{1/2}^{90}$, were 220 μM and 3.2 mM, respectively, in NaCl buffer and 100 μM and 1.7 mM in KCl buffer. Since the exact quantitative relationship of ATPase inhibition to cell lysis depends on several unknown factors (e.g., membrane permeability to the inhibitors and the minimum level of residual ATPase activity necessary to maintain cellular osmotic stability), the closeness of the $K_{1/2}^{90}$ values to the K_I's for ATPase inhibition *in vitro* (170 μM and ∿ 2.2 mM, respectively, for PCMBS and NEM) can be considered consistent with the proposal that a functional membrane ATPase is essential for maintenance of cellular osmotic stability in alkali cation-containing media.

Conclusions

The results just discussed, and the specific activation of the ATPase *in vitro* by Na^+ ions, suggest that the (Mg^{2+},Na^+)-ATPase of the *A. laidlawii* B membrane functions in osmotic regulation as does the (Mg^{2+},Na^+,K^+)-ATPase of eukaryotes (Christensen, 1975). This is the first demonstration of an osmoregulatory alkali cation pump in a prokaryote,

and it may be of considerable evolutionary interest. The failure of K^+ ions to stimulate or to modify the Na^+ effect on the ATPase *in vitro* is perplexing in view of the findings that cells can grow on a low-Na^+ medium (Cho and Morowitz, 1969) and are stable in KCl buffers in the presence of glucose (this study). Three possible explanations may be advanced to explain this apparent inconsistency:

(1) The ATPase acts as a K^+ pump *in vivo* but, for any of a variety of possible reasons, fails to exhibit any K^+ effect on phosphohydrolase activity in my assay system.

(2) The ATPase can transport H^+ as well as Na^+; the energy of a transmembrane proton electrochemical gradient generated by the ATPase can be coupled to the extrusion of K^+ by a specific transporter. This proposal agrees with the suggestion of Tarshis and Kapitanov (1978) that the ATPase generates a protonmotive force for energization of active solute uptake as the $BF_o \cdot F_1$-ATPases do in higher bacteria (Harold, 1977).

(3) The ATPase can generate a significant Na^+ electrochemical potential gradient by extruding Na^+ even when the extracellular Na^+ concentration is low (1 - 10 mM). This Na^+ gradient energy could then be coupled to K^+ extrusion. This explanation seems quite plausible when one considers that in *E. coli*, an entirely similar system energizes the active transport of many solutes by extruding protons into a medium whose H^+ concentration is usually < 1 μM.

The first explanation is intellectually dissatisfying but quite plausible; the second, containing a similar *deus ex machina* element, is scarcely more pleasing. The third explanation, while based largely on demonstrable properties of the ATPase, still requires a postulated mechanism for coupling Na^+ and K^+ movements (an antiporter?). Therefore, the

exact role of the ATPase in the movement of K^+ (and of H^+) out of the *A. laidlawii* cell must remain an open question at present.

CHAPTER 7

DEPENDENCE OF THE MEMBRANE ATPase ACTIVITY ON THE LIPID PHYSICAL STATE IN A. laidlawii B

Background

Having established certain basic properties of the membrane lipid phase in A. laidlawii B grown with avidin and exogenous fatty acids, and having characterized the basic kinetic and functional properties of a membrane enzyme, the (Na^+,Mg^{2+})-ATPase, in this organism, we now turn to an investigation of the relationship(s) between these two sets of properties. Our basic tools in these studies will be the Arrhenius plot (of the logarithm of the enzyme's V_{max} vs. reciprocal absolute temperature) and DTA of the membrane lipids, combining theoretical and experimental results to shed light on the nature of the interaction between the enzyme and its surrounding lipid matrix, particularly in respect to the manner in which the lipid physical state affects the enzyme activity.

If asked to name some common 'membrane probe techniques', most membrane workers would readily mention lipid spin label methods, fluorescence depolarization, nuclear magnetic resonance, and probably a few other spectroscopic, optical or calorimetric methods. A much smaller number of workers would likely mention 'enzyme kinetics' in this regard, for although it is at least tacitly assumed that many membrane enzyme activities are sensitive to the physical state of the lipid phase (Linden and Fox, 1975; Cronan and Gelmann, 1975; Sandermann, 1978), few rigorous efforts have been made to relate these two membrane properties on a mechanistic or molecular level. Certain basic questions must be asked (and answered) in order to exploit the kinetic properties of membrane enzyme

activities (and particularly their temperature dependences) to obtain fundamental information regarding the physical properties of the membrane lipids, and regarding the nature and functional consequences of the interactions of enzyme and lipid molecules in the membrane. First, what is the behavior of the enzyme 'probe' molecule in the membrane; e.g., does it prefer one lipid environment (gel or liquid-crystalline) over another, and does it change its conformation as a function of temperature or the state of the associated lipid? Secondly, how does the 'signal' (e.g., a measured V_{max}, a K_m, etc.) obtained from the 'probe' (enzyme) relate to the behavior and environment of the enzyme in the membrane? While an exhaustive study of all aspects of the enzyme's behavior (solvent exposure, rotational motion, etc.) as a function of temperature and lipid environment would undoubtedly yield answers to the above questions, such a study would require an enormous amount of effort and would not generally be feasible if the enzyme had to be studied *in situ* (i.e., without removing it from its native membrane environment).

A simpler alternative to the experimental program just described for the study of the relationship of enzyme activities to the membrane lipid physical properties would be the following: controlled alterations in the membrane lipid composition would be induced without disrupting the membrane (preferably by *in vivo* lipid manipulation) and the resultant changes in the lipid physical properties (determined by one or two basic methods) would be correlated with any accompanying changes in the enzyme's kinetic properties. The most frequently studied enzyme property in the studies that have followed this latter pattern to date has been the temperature dependence of its activity. Over the last few years, it has become a common practice for membrane workers to generate Arrhenius plots

from their rate-temperature data for a given system, identify nonlinear behavior (indicating a non-constant enthalpy of activation) and attribute such behavior to a membrane 'phase transition' or 'lateral phase separation (for example, see Dockter et al., 1978), sometimes with only minimal evidence that the latter phenomena are in fact occurring in the membrane. A few studies have attempted to quantitatively account for the nonlinear Arrhenius plots observed for certain membrane protein-mediated processes, such as the transport of β-galactosides or β-glucosides (Thilo et al., 1977) in E. coli, making specific mechanistic proposals as to the basis of such nonlinear plots. In general, however, little if any attempt is made in most cases to explain the nonlinearity of Arrhenius plots for membrane enzymes (most notably the biphasic linear plots frequently observed) in terms of molecular processes more precise than 'discontinuous phase changes' or 'lateral phase separations'. In the work to be described here, I have developed a theoretical framework for the analysis of nonlinear Arrhenius plots for membrane-related rate processes and have used this theoretical basis to analyze the temperature dependence of the A. laidlawii membrane ATPase activity as a function of the membrane lipid acyl chain composition and phase transition temperature.

Theory

The theoretical framework to be considered here is part of a larger and more general one which we have discussed elsewhere (Silvius and McElhaney, 1979c). I shall be concerned here only with those models which can reasonably quantitatively account for the observed temperature dependence of the A. laidlawii membrane ATPase activity in various lipid

environments. Let us assume, as a simple yet general model, that a lipid phase transition can somehow induce a change in the state of a membrane enzyme. State 1 and state 2 in general will have different activation free energies $\Delta G^{\ddagger}$, and by the use of a standard result from absolute reaction rate theory (Johnson et al., 1974), we can readily obtain the rate equation

$$(\text{Rate}) = (\frac{NkKT}{h})(f_1 \exp(-\Delta G_1^{\ddagger}/RT) + f_2 \exp(-\Delta G_2^{\ddagger}/RT)) \qquad [7\text{-}1]$$

where f_1 and f_2 are the fractions of the total enzyme (N molecules) in states 1 and 2, T is the absolute temperature, K is a 'transmission coefficient' usually set equal to unity, and k and h are the Boltzmann and Planck constants, respectively. To transform this equation into one useful in the analysis of Arrhenius plots, we must determine the temperature dependence of f_1 and f_2. The simplest way of doing this is to adopt a standard free-energy expression for the populations of the two states, $f_2/f_1 = \exp(-\Delta G_{21}/RT)$, which can be readily transformed into the equations

$$f_1 = (1 + \exp(-\Delta G_{21}/RT))^{-1} \qquad [7\text{-}2a]$$

$$f_2 = (1 + \exp(\Delta G_{21}/RT))^{-1} \qquad [7\text{-}2b]$$

where ΔG_{21} is the free-energy change on passing from state 1 to state 2. Using these expressions in combination with equation [7-1], and taking natural logarithms, we obtain the general expression

$$(\ln \text{Rate}) = \ln(\frac{NkK}{h}) + \ln(T) - (\Delta G_2^{\ddagger}/RT) - \ln(1 + \exp(\Delta G_{21}/RT)) + \ln(1 + \exp((\Delta G_{21} + \Delta(\Delta G^{\ddagger}))/RT) \qquad [7\text{-}3]$$

where $\Delta(\Delta G^{\ddagger}) = \Delta G_2^{\ddagger} - \Delta G_1^{\ddagger}$. Equation [7-3] can be expressed in an ultimately more useful form if we express the free energy terms as sums of enthalpic and entropic components, or as a function of an enthalpic term and a 'characteristic temperature', T_o, at which the free energy tends to zero:

$$\Delta G = \Delta H - T\Delta S = \Delta H(1 - T/T_o) \qquad [7\text{-}4]$$

The last identity results if ΔH and ΔS are constant or nearly so over the temperature range studied, in which case $T_o = \Delta H/\Delta S$. It should be noted that if T_o falls outside of the temperature range studied, it may not represent the true temperature at which $\Delta G = 0$ (for example, if ΔH and ΔS can only reasonably be treated as constant over a narrow range of experimental temperatures). However, T_o remains a convenient parameter to use in analyzing the shape of Arrhenius plots, as we shall see below.

I now introduce a new function, called the 'Eb' (for 'exponentially breaking') function, which is defined as follows:

$$Eb(\Delta H^*, T_o; T) \equiv \ln(1 + \exp(\frac{\Delta H^*}{R}(1/T - 1/T_o)) \qquad [7\text{-}5]$$

Using this functional symbolism and the free-energy representations just discussed, equation [7-3] takes on its final form [7-6]:

$$\ln(\text{Rate}) = \ln(\frac{NkK}{h}) + \ln(T) - (\Delta G_2^{\ddagger}/RT) - Eb(\Delta H_{21}, T_1; T) + Eb(\Delta H_{21} + \Delta(\Delta H^{\ddagger}), T_2; T) \qquad [7\text{-}6]$$

where $T_1 = \Delta H_{21}/\Delta S_{21}$ and $T_2 = (\Delta H_{21} + \Delta(\Delta H^{\ddagger}))/(\Delta S_{21} + \Delta(\Delta S^{\ddagger}))$. While equation [7-6] may appear to be rather intimidating, we note that it consists of only a few significant elements: (1) a constant term, $\ln(NkK/h) + \Delta S_2^{\ddagger}/R$; (2) a slowly varying logarithmic term, $\ln(T)$;

(3) a linear term in (1/T), $\Delta H_2^{\ddagger}/RT$; and (4) the differences of two Eb functions whose enthalpy parameters and characteristic temperatures will in general be different. To translate equation [7-6] into a quantitative description of the behavior of an Arrhenius plot, we can ignore terms (1) and (2), which are constant or nearly so over the physiological temperature range and furnish little information unless N, the number of active enzyme molecules, is known. We then retain only terms (3) and (4) in our modified equation [7-6], plus an arbitrary constant to properly scale the rates predicted from equation [7-6] to the experimental data. To obtain a useful qualitative description of the behavior of an Arrhenius plot generated from equation [7-6], we must consider the properties of the Eb function.

The most significant features of the Eb function for our analysis are the following:

(1) The limiting slopes of the Eb function $Eb(\Delta H^*, T_o; T)$ on an Arrhenius plot are $\Delta H^*/R$ and zero. The Arrhenius plot of an Eb function is always concave upward, and that of its negative is always concave downward.

(2) The Eb function on an Arrhenius plot shows a region of intermediate slope around T_o. Extrapolation of the limiting straight-line portions of the plot toward the 'break' region gives an intersection of the limiting tangents at T_o.

(3) The 'break' of the Eb function is progressively sharper as ΔH^* increases. However, Arrhenius plots of the Eb function for low ΔH^* values will be relatively gently curving and can often be fitted to within a very modest experimental error (<5%) by two straight lines.

Some representative Arrhenius plots of the negative of the Eb function are shown in Figure 18.

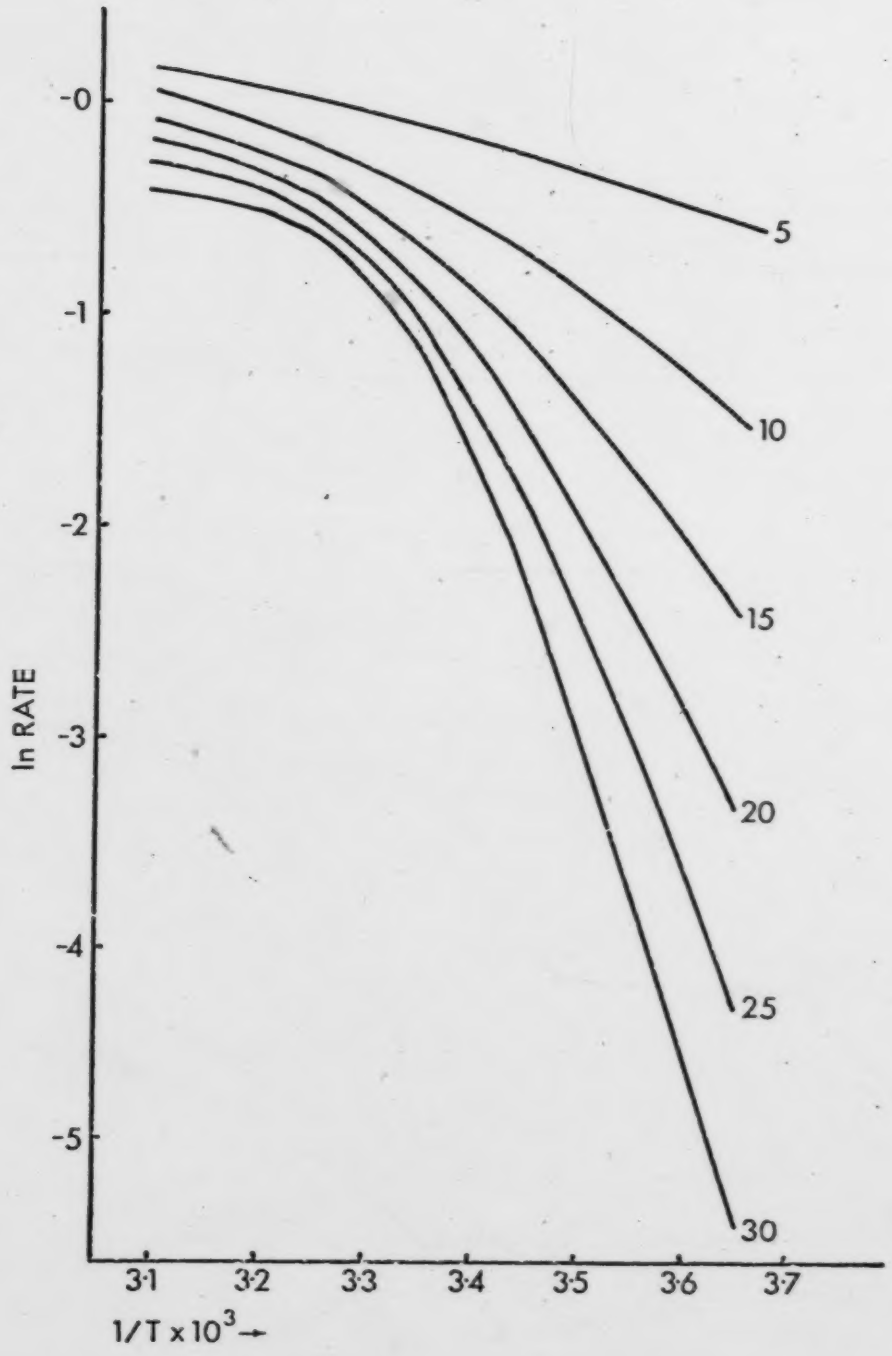

Figure 18. Arrhenius-type plots of the negative of the Eb function, $Eb(\Delta H^*, T_o; T) \equiv \ln(1 + \exp((\Delta H^*/R)(1/T - 1/T_o)))$, for $T_o = 300°K$ and ΔH^* variable (given in kcal mole^{-1} next to the graphs).

If we apply the considerations just discussed to an analysis of the shape of the Arrhenius plot generated by equation [7-6], we come to the following conclusions:

(1) The Arrhenius plot will have limiting slopes of $-\Delta H_1^{\ddagger}/R$ and $-\Delta H_2^{\ddagger}/R$.

(2) The Arrhenius plot will have two 'breaks', one of upward concavity and one of downward concavity, unless the T_o values of the two Eb functions are the same.

(3) The midpoint of the 'break' region of downward concavity will be the temperature at which $\Delta G_{21} = 0$ and hence equal amounts of the protein are in states 1 and 2.

(4) The 'break' regions of the Arrhenius plot will in general be curved, unless $T_1 = T_2$ in equation [7-6], in which case a single sharp break will be seen. However, if the changes in slope around the breaks are modest ($\Delta H^* < 15$ kcal mole^{-1}), the curvature may be lost in the scattering of the data and the graph may approximate a set of straight lines. A sample of an Arrhenius plot generated from equation [7-6] is shown in Figure 19.

A few other theoretical points will be useful in our analysis of the Arrhenius plots for the A. laidlawii ATPase. First, if we assume that state 1 of the enzyme in the model above is wholly inactive, equation [7-6] reduces to the expression

$$\ln(\text{Rate}) = \ln(\frac{NkTK}{h}) + \ln(T) - \Delta G_2^{\ddagger}/RT - Eb(\Delta H_{21}, T_o; T) \qquad [7\text{-}7]$$

where $\Delta G_2^{\ddagger}$ can be any function of temperature but ΔG_{21} is assumed to vary linearly with temperature. In this case, the limiting tangent to the Arrhenius plot in its steeply sloping portion will intersect the graph of the temperature-activity relationship for the active form of the enzyme

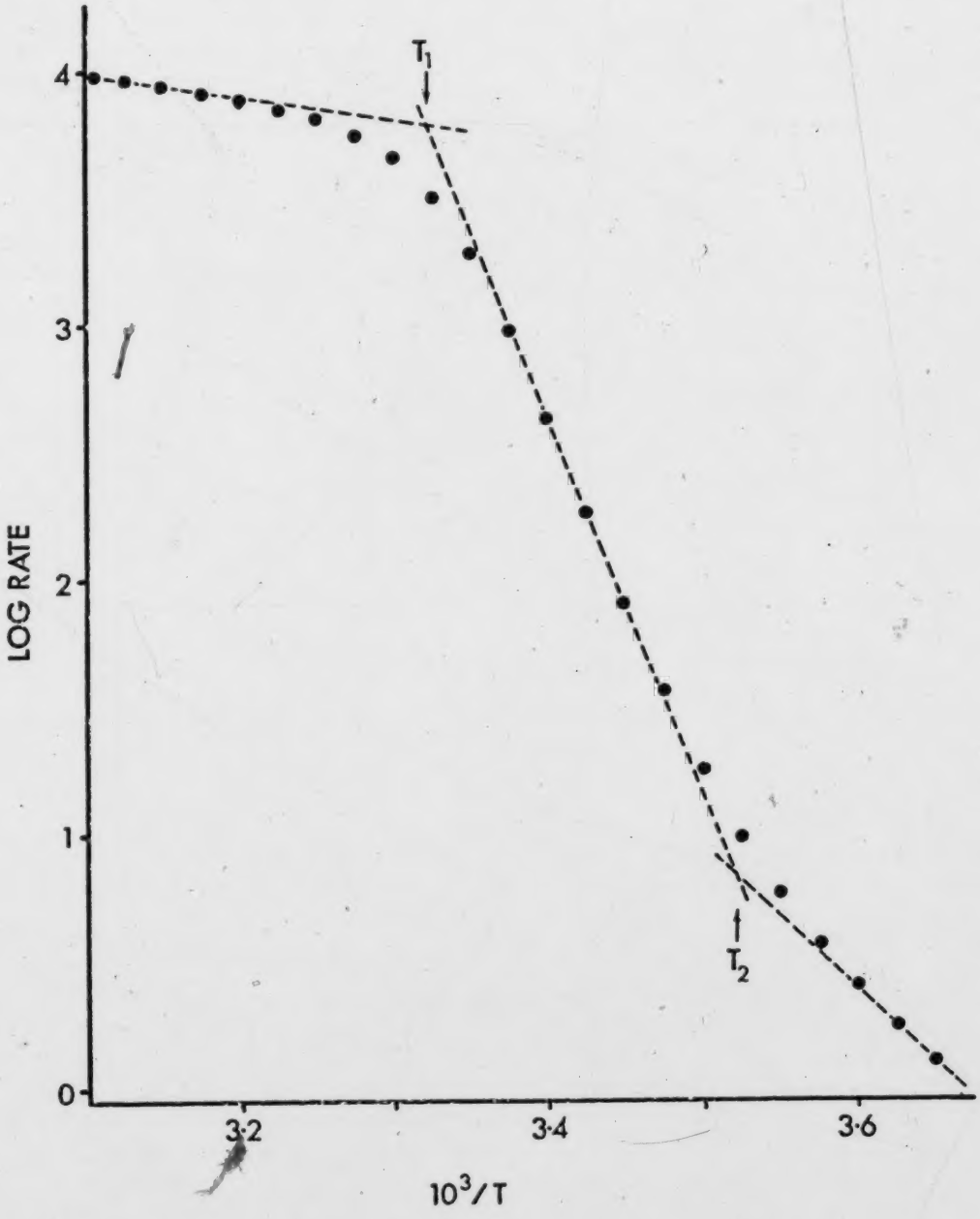

Figure 19. A theoretical Arrhenius plot, generated from equation [7-6] in the text using the following parameters: T_1 = 300°K, T_2 = 285°K, $\Delta H_2^{\ddagger}$ = 5 kcal mole^{-1}, ΔH_{21} = 80 kcal mole^{-1}, $\Delta H_1^{\ddagger}$ = 50 kcal mole^{-1}. The dashed lines represent the three linear phases of the graph, and T_1 and T_2 are indicated on the graph.

at T_o, the temperature at which the enzyme is equally distributed between states 1 and 2 (see T_1 in Figure 19). In equations [7-6] or [7-7], the value of ΔG_{21} will be physically interpretable in different ways, depending on the details of the physical model used. For example, if the enzyme undergoes a conformational change, ΔG_{21} is the free energy of that change. If the enzyme partitions between the gel- and liquid-crystalline lipid phases and has different activation free energies in the two phases, $\Delta G_{21} = \Delta G_p + \Delta G_{eff}$, where ΔG_p is the free energy of partition of the enzyme from the gel to the liquid-crystalline phase, and ΔG_{eff} is an 'effective phase transition free energy' that describes the progress of the lipid phase transition according to the equation

$$\frac{f_{lc}}{f_g} = \exp(-\Delta G_{eff}/RT) \qquad [7\text{-}8]$$

where f_{lc} and f_g are the fractions of the total lipid in the liquid-crystalline and gel phases, respectively. Finally, if the boundary lipid surrounding the enzyme undergoes a type of phase change and thereby changes the enzyme's activation parameters, ΔG_{eff} in the last model is replaced by an effective free energy change <u>for the boundary lipids</u>, which will in general have smaller enthalpic and entropic components than does the ΔG_{eff} value for the bulk lipids. This last point arises from theoretical considerations (Marčelja, 1976) of the phase behavior of protein-associated lipids in the presence of 'free' (non-protein-associated) lipids which undergo a cooperative phase transition. As we shall see, if we compare the values of $T_1(T_o)$ and ΔH_{21}, derived from Arrhenius plot analyses using equations [7-6] and [7-7], with the values of T_{AL} (the phase transition midpoint) and of ΔH_{eff} determined by DTA of

membrane lipids, we can obtain considerable information regarding the physical basis for the effects of the lipid physical state on the activity of the A. laidlawii membrane ATPase.

Experimental Results and Comparison with Theory

My initial studies of the temperature dependence of the A. laidlawii B membrane ATPase activity used varying substrate concentrations at each temperature so that both V_{max} and K_m could be determined. However, as this method required collection and analysis of a large number of samples at each temperature, it was very difficult to study more than 10 temperatures in a given membrane preparation over a reasonable length of time (8 - 12 hours). Since, as was discussed in the last chapter, the temperature variation of K_m is the same within experimental error in all membrane preparations studied, regardless of the lipid fatty acid composition, I used a simpler procedure to measure the temperature dependence of the ATPase reaction in my later experiments. In this simpler procedure, I measured the rate of reaction (as P_i release) at 1 mM ATP in duplicate at each temperature, repeating any runs in which the best-fit slopes for the time courses of reaction in the two samples disagreed by more than 3%. I then converted the velocity at 1 mM ATP to a V_{max} by reference to an average of the pooled estimates of K_m derived from a number of earlier experiments (Figure 16 of the last chapter provides a sample of the K_m data collected), and the corrected V_{max} data were then plotted as a standard Arrhenius plot. The magnitude of the K_m correction is generally small in comparison with the large variation of V_{max} with temperature, particularly in the low-temperature region (<25°) where most of our attention will be focused. By measuring a reaction velocity at a

relatively high ATP concentration (1 mM), the initial velocity (i.e., the velocity in the linear region of the time course) of the reaction could easily be determined without the necessity of maintaining a low total release of P_i (which reduces the accuracy of rate measurements) in order to avoid ATP depletion. As well, the procedure I have just described allowed me to determine the rate of the ATPase reaction at up to 22 temperatures, to an estimated average error of <3%, in a single day's work (which minimized the problem of slow inactivation or modification of the ATPase or its membrane environment). Arrhenius plots determined by this simpler procedure agree very well with plots determined using varying ATP concentrations, both for fatty acid-homogeneous membranes (14:0i, 16:0i) and for 'fatty acid-enriched' membranes from cells grown with a fatty acid (16:0, 16:0i, 18:1tΔ^9) but without avidin. The Arrhenius plots to be discussed below were all determined using 1 mM ATP because more temperature points were collected and because the average error of rate determinations was less when this procedure was used.

Activity of the ATPase Associated with Liquid-Crystalline Lipids - Results derived from DTA measurements (Chapter 4) show that membranes whose lipids are homogeneous in certain fatty acids should contain a very high proportion (>95%) of liquid-crystalline lipids even at 0°C. To determine the 'intrinsic' temperature dependence of the ATPase activity in the absence of a lipid phase transition, I determined the temperature dependence of this activity in membranes made homogeneous in cis-vaccenic (18:1cΔ^{11}) or anteisopentadecanoic acid, both of which give lipid phase transitions centered well below 0°C. The resulting Arrhenius plots are shown superimposed in Figure 20, from which it can be seen that the overall temperature dependence of the enzyme activity is essentially identical

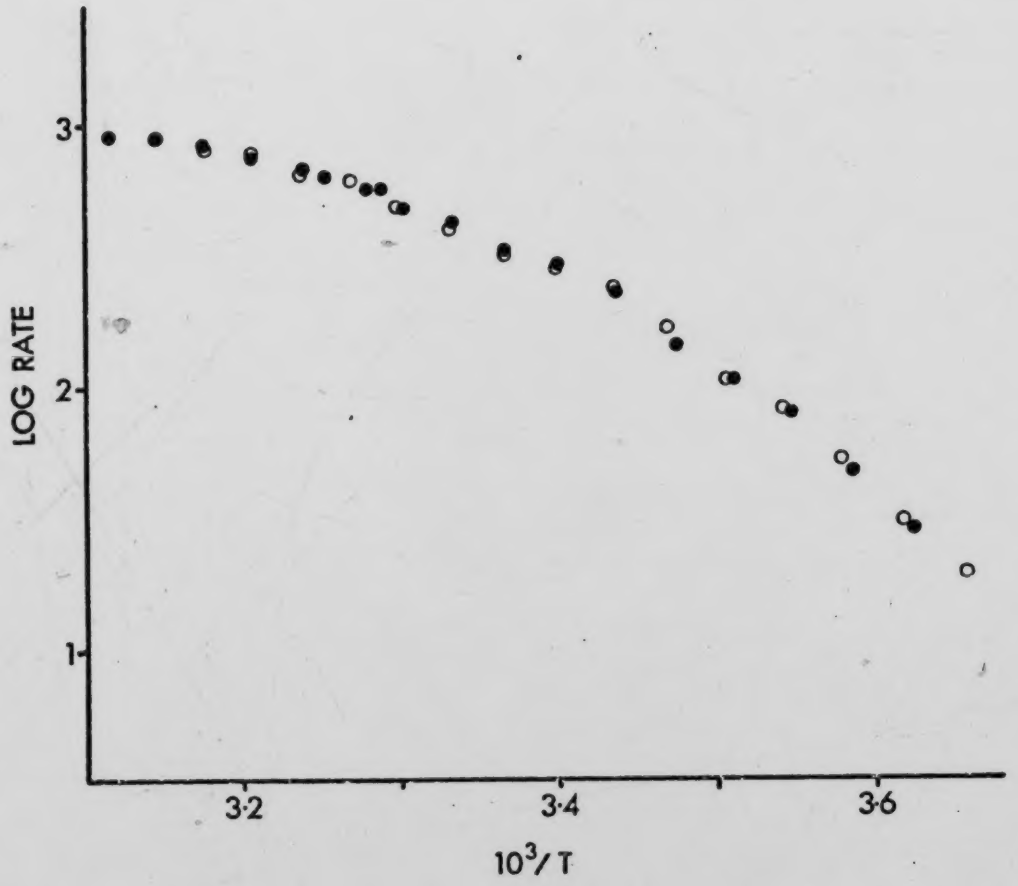

Figure 20. Superimposed Arrhenius plots for the ATPase activity in two types of membranes, having quite different fatty acyl composition but both containing essentially purely liquid-crystalline lipids above 0°C. The (log Rate) values given are the logarithms of V_{max} values, in nmole P_i released per min per mg membrane protein, determined as described in the text for (●) anteisopentadecanoate- and (○) cis-vaccenate-homogeneous membranes.

in the two types of membranes. Since cis-vaccenic and anteisopentadecanoic acids have quite different structures (cis-vaccenate has a long chain with an internal distortion from an all-trans polymethylene arrangement, while anteisopentadecanoate has a considerably shorter chain with a terminal distortion), it appears that the enzyme activity is not appreciably sensitive to the fatty acyl chain structure of the lipids around it so long as the lipids are in the liquid-crystalline state.

Can we quantitatively account for the shape of the rate-temperature relationship shown in Figure 20? Models postulating a temperature-dependent change in the enzyme conformation or rate-limiting step do not account for the overall shape and slope of the curve as well as does a model recently proposed by Sturtevant for enzymic reactions in general (Sturtevant and Mateo, 1978), in which a finite and constant heat capacity of activation, $\Delta C_p^{\ddagger}$, is assumed to cause $\Delta H^{\ddagger}$ to be a linear function of temperature, thereby causing the Arrhenius plot to be continuously curving. In fact, when I took a series of tangents to one of the Arrhenius plots of Figure 20 and calculated the apparent activation enthalpy as a function of temperature (using the equation $\Delta H^{\ddagger} = -R(d(\ln \text{Rate})/d(1/T)) - RT$ (Raison, 1973)), the calculated values of $\Delta H^{\ddagger}$ were a nearly linear function of temperature. This is the result expected from the model just noted, based on the relationship $\Delta H^{\ddagger} = \Delta H_o^{\ddagger} + \Delta C_p^{\ddagger}(T-T_o)$, where the value of $\Delta H^{\ddagger}$ is $\Delta H_o^{\ddagger}$ at some reference temperature T_o. From the plot of $\Delta H^{\ddagger}$ vs. T, I calculated a value of $\Delta C_p^{\ddagger}$ of -550 cal $mole^{-1}$ deg^{-1}, and the Arrhenius plot for the ATPase in anteisopentadecanoate-homogeneous membranes can in fact be well fit using a $\Delta C_p^{\ddagger}$ value of -515 cal mol^{-1} deg^{-1} in the equation

$$\ln \text{Rate} = K - (\Delta H_o^{\ddagger}/RT) - \Delta Cp^{\ddagger}/R(\ln(T_o/T) - T_o/T) \qquad [7\text{-}9]$$

where K is an arbitrary scaling constant, as is shown in Figure 21. Therefore, the appreciable nonlinearity of the Arrhenius plot for the ATPase in a liquid-crystalline lipid environment appears to be best explained by a finite heat capacity of activation rather than by a change in the state or rate-limiting step of the enzyme.

Effect of a Lipid Phase Transition on the ATPase Activity - To study the effect of a lipid phase transition on the ATPase activity, I examined the Arrhenius plots for the ATPase in a series of membranes of varying fatty acid composition whose lipid phase transitions occur well above 0°C. One such Arrhenius plot is shown in Figure 22, where it can be seen that the plot is curvilinear and does not have a true 'bilinear' form as has previously been reported for this enzyme, by us and by other workers (DeKruyff et al., 1973; Jinks et al., 1978), as well as for many other membrane-bound enzymes (Raison, 1973; Linden and Fox, 1975). In fact, the Arrhenius plot shown can be reasonably well described by the sum of a linear component and a single Eb function, which is the functional form that equation [7-7] predicts if only one of the characteristic temperature parameters, T_1 and T_2, fall within the temperature range studied. The fact that the 'break' region can be fit by a single Eb function cannot be explained by a model that suggests that the enzyme is converting from a high-activation enthalpy form below the 'break' temperature, T_1, to a low-activation enthalpy form above it, and that the slope of the Arrhenius plot below T_1 represents the activation enthalpy of the low-temperature form. If this were true, the sharpness of the 'break' should be considerably greater than that for a single Eb function and should rapidly increase as the sharpness of the membrane lipid phase transition increased. These predictions were not fulfilled by my experi-

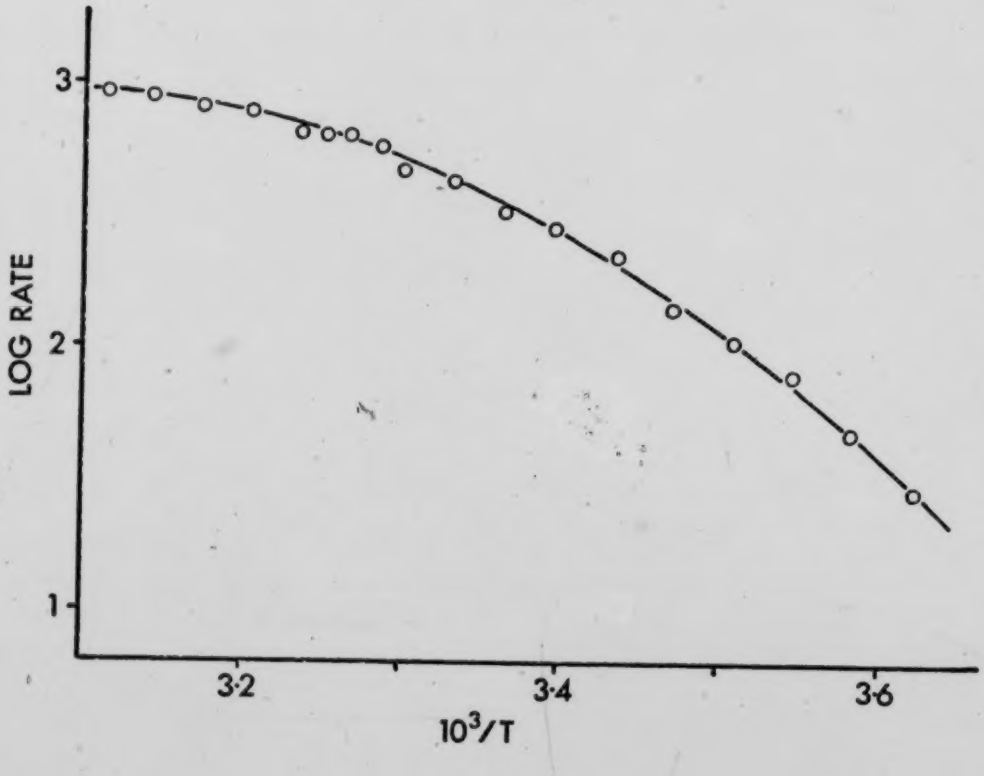

Figure 21. Description of the temperature dependence of the ATPase activity in anteisopentadecanoate-homogeneous membranes in terms of a finite heat capacity of activation, $\Delta C_p^{\ddagger}$. Experimental data points have been fit by the equation:

$$(\log \text{Rate}) = K + (\Delta C_p^{\ddagger}/R) \ln T - \Delta H_o^{\ddagger}/RT - (\Delta C_p^{\ddagger}/R)(T_o/T)$$

with $\Delta C_p^{\ddagger} = -515$ cal mole^{-1} deg^{-1}, T in °K, and $\Delta H_o^{\ddagger} = 13.8$ kcal mole^{-1} at $T_o = 300$°K.

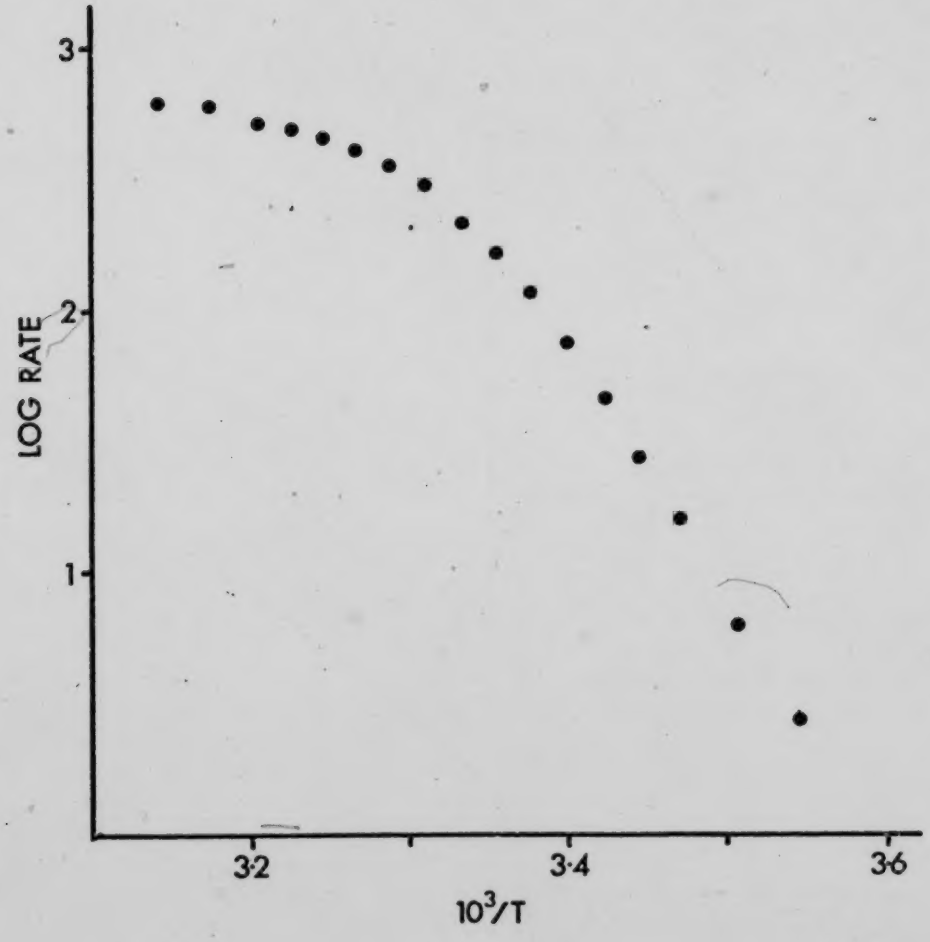

Figure 22. An Arrhenius plot for the ATPase activity (as V_{max}, in nmol P_i released per mg membrane protein per min) in membranes of cells grown with avidin (2 mg/l) plus 0.06 mM each elaidate ($18:1t\Delta^9$) and palmitate (16:0). Experimental details are given in the text.

mental results. The characteristic temperature, T_1, determined for Figure 22 is 24.8°C, while the midpoint of the lipid phase transition for these membranes (whose lipids contain roughly 50% each of palmitate (16:0) and elaidate (18:1tΔ^9)) is found to be 26.9° by DTA. In general, as we shall see later, the characteristic temperature T_1 is found to vary systematically with the midpoint of the lipid phase transition, T_{AL}, although the value of T_1 is always somewhat lower than is T_{AL}.

Some of the Arrhenius plots observed for the ATPase membranes exhibiting a lipid phase transition at physiological temperatures showed a feature not apparent in Figure 22, namely a second, concave upward 'break' at low temperatures and very low activities. An example of an Arrhenius plot containing this second 'break' is shown in Figure 23, where isoheptadecanoate-homogeneous membranes were studied. A low-temperature break of upward concavity could have either of two possible physical bases: either the ATPase itself is active in its low-temperature form (called state 1 in deriving equation [7-7]), or low levels of a contaminating ATP hydrolytic activity are becoming exposed when the (Na^+,Mg^{2+})-ATPase activity is reduced to very low levels. To distinguish between these two possibilities, I studied the relative thermolability of the high- and low-temperature ATPase activities in isoheptadecanoate-homogeneous membranes by exposing membrane samples to various high temperatures (50°C - 65°C in 5° steps) for 5 min, then cooling the samples and assaying their ATPase activities at 5°C and at 37°C. The 5° activity showed a steady decline with increasing preincubation temperature (73%, 49%, 39% and 26% of the control activity remained after 5 minutes at 50°, 55°, 60° and 65°, respectively), while the 37° activity was more stable up to 60° but showed a very steep decline in activity above 60° (79%, 71%, 45% and <3% of

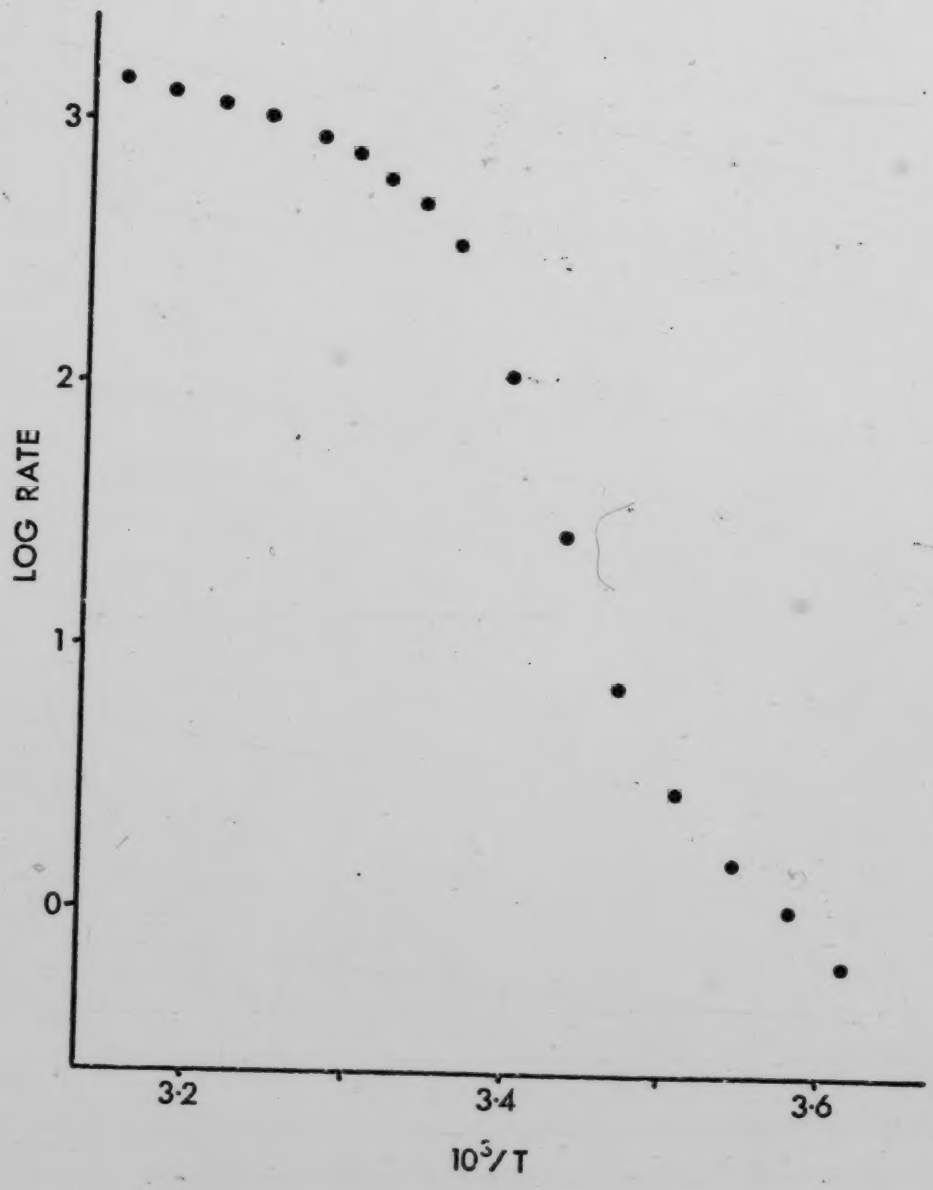

Figure 23. An Arrhenius plot for the ATPase activity in membranes of cells grown with avidin (2 mg/ml) plus 0.12 mM isoheptadecanoic acid. Experimental details are as for Figure 22.

control activity remained after 5 minutes at 50°, 55°, 60° and 65°, respectively). Therefore, the low-temperature break in the ATPase Arrhenius plot seems to represent the emergence of a low-level ATP hydrolytic activity distinct from that of the (Na^+,Mg^{2+})-ATPase, and it was not studied further. I would note in passing that while Thilo et al. (1977) have recently proposed that membrane enzymes in general may be active when surrounded by gel-phase lipids, the systems on which they base their proposal, the β-glucoside and β-galactoside transport systems of E. coli, exhibit an appreciable 'leakage' component due to passive permeation of substrate and possibly to the action of other sugar transport systems. It is entirely possible, therefore, that the low-temperature activities reported by these authors are not catalyzed by the transport systems which catalyze the high-temperature activities, and that the lower Arrhenius plot 'breaks' reported by them are artifactual, just as they appear to be for the A. laidlawii B membrane ATPase.

To supplement the qualitative observations just discussed regarding the shape of the ATPase Arrhenius plots for membranes undergoing a lipid phase transition, I now wish to consider the Arrhenius plots in a more quantitative manner. If we ignore the low-temperature activity (which generally is significant only when the total ATPase activity has decreased to roughly 2 - 3 nmole min^{-1} mg^{-1} or less), and consider the enzyme's temperature-activity relationship when the enzyme is associated with liquid-crystalline lipid, we can describe the upper 'break' and the region around it by the equation

$$\ln \text{Rate} = F(T) - Eb(\Delta H_{21}, T_o; T) \qquad [7\text{-}10]$$

where F(T), given by equation [7-9], describes the enzyme activity in the

active state 2 (including a finite $\Delta C_p^{\ddagger}$ value), and ΔH_{21} is again the 'effective enthalpy of transition' between the two states and $T_o = (\Delta H_{21}/\Delta S_{21})$. This equation is appropriate if the ATPase is inactive in state 1, as seems to be the case (vide supra). As we see in Figure 24, equation [7-10] does in fact provide a very good quantitative description of the Arrhenius plot observed for the ATPase in a membrane which undergoes a lipid phase transition in the physiological temperature range. In Table 12, I have summarized the values of T_o obtained by analyzing a variety of Arrhenius plots in terms of equation [7-10], as well as the midpoint temperatures, T_{AL}, of the membrane lipid phase transitions in the preparations thus analyzed. It can be seen that T_o and T_{AL} are clearly correlated, although T_o can lie several degrees below T_{AL} in certain cases, notably when monounsaturated fatty acid supplements are used. Therefore, the upper break in the Arrhenius plot for the ATPase clearly appears to result from a lipid phase transition-induced change in the state of the enzyme.

A lipid-induced change in the state of the ATPase could conceivably come about in one of two ways. First, the enzyme molecules could partition between gel and liquid-crystalline lipid domains with free energy ΔG_p, which in general would be a function of temperature, as would the phase partition coefficient derived from it. In this case, ΔH_{21} in equation [7-9] would be equal to $\Delta H_p + \Delta H_{eff}$, where ΔH_{eff} defines the sharpness of the lipid phase transition, as I have discussed in the last section. Secondly, the enzyme's boundary lipids could themselves undergo a change in state which was driven by the bulk phase transition but which would probably be of lower cooperativity and hence would have a lower ΔH_{eff} than did the bulk lipid phase transition. To decide between these

Table 12

Lipid Phase Transition Midpoint Temperatures (T_c) and ATPase Equidistribution Temperatures (T_o), Determined for Various Membrane Preparations by DTA and by Arrhenius Plot Analysis, Respectively. Details of the analyses are described in the text.

Growth Supplement [a]	T_c (°C)	T_o (°C)
None	33.8	30.9
Palmitate	31.2	27.7
Palmitate/cholesterol	30.7	25.3
Isopalmitate	25.8	22.3
Elaidate	28.7	22.4
Anteisopentadecanoate/avidin	-14.5	- [b]
Cis-vaccenate/avidin	-8.5	- [b]
Isomyristate/avidin	10.1	7.2
Isopentadecanoate/avidin	14.8	13.5
Trans-vaccenate/avidin	20.0	14.0
Elaidate/avidin	20.1	14.9
Isopalmitate/avidin	21.8	20.9
Palmitate/elaidate/avidin	26.9	24.8
Cis-15-octadecenoate/avidin	27.6	20.7
Isoheptadecanoate/avidin	28.8	25.3
Palmitate/myristate/avidin	32.9	30.7

[a] Fatty acids were added to 0.12 mM, avidin to 2 mg ml in the culture medium.

[b] No break was seen above 0°C in these Arrhenius plots.

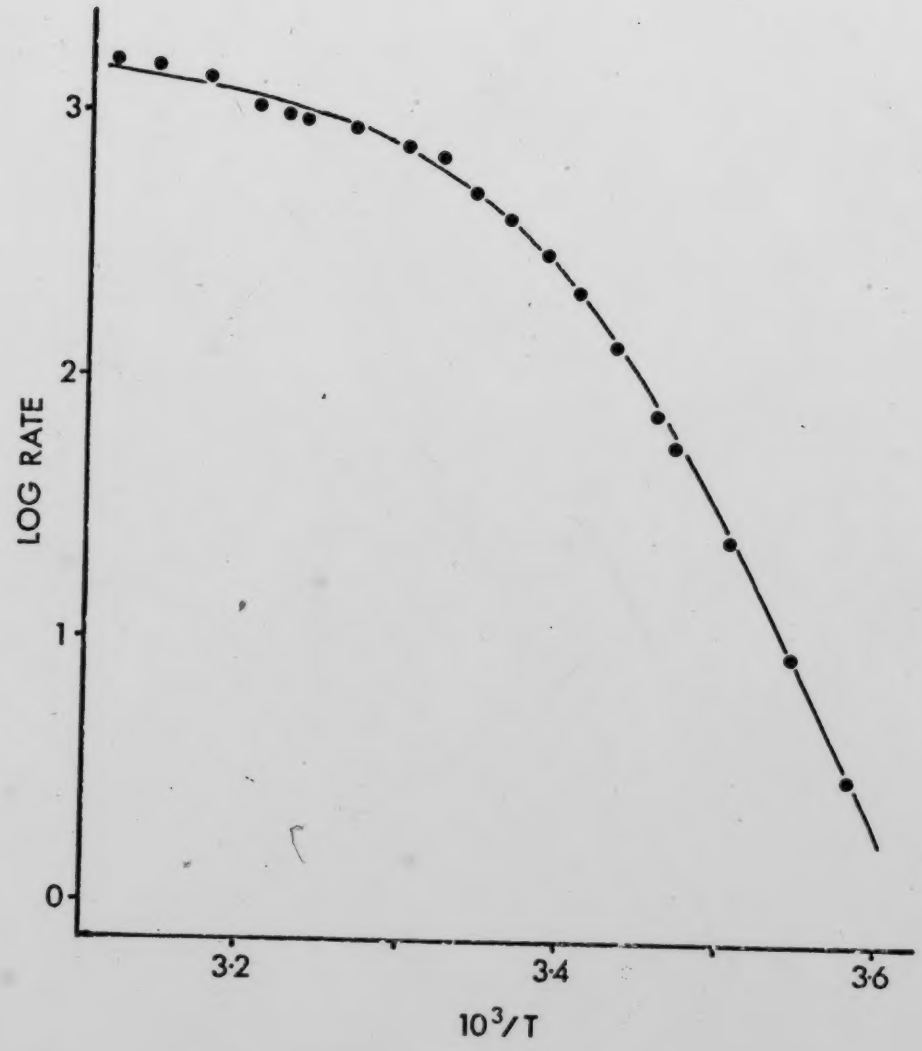

Figure 24. Description of the Arrhenius plot for the ATPase activity in cis-15-octadecenoate-homogeneous membranes by equation [7-10] (solid line). The following parameters were used in equation [7-10]: ΔH_{21} = 36 kcal mole^{-1}, T_o = 20.7°C.

two possibilities, I determined ΔH_{21} from a series of Arrhenius plots and plotted the experimental values vs. the values of ΔH_{eff} determined by DTA for the corresponding membrane lipid phase transitions. The latter values were determined from the equation

$$\Delta H_{eff} = RT_{AL}^{2}(\ln 81)/\delta T(10\rightarrow 90) \qquad [7\text{-}11]$$

where R = 1.987 cal mole^{-1} deg^{-1} and $\delta T(10\rightarrow 90)$ is the temperature range required for the transition to pass from 10% to 90% of completion, as it was defined in Chapter 4. Equation [7-11] is derived from equation [7-8] by setting f_{lc}/f_g equal first to 9, then to 1/9, and dividing both sides of the first equation (using $f_{lc}/f_g = 9$) by the same sides of the second (using $f_{lc}/f_g = 1/9$), then taking the logarithms of both sides and rearranging. The plot of ΔH_{21} vs. ΔH_{eff}, shown in Figure 25, yields a correlation coefficient of 0.90 and a best-fit slope of 0.27, indicating that while ΔH_{21} is proportional to ΔH_{eff}, the coefficient of proportionality is much less than unity. The value of the y-intercept (roughly 10 kcal mole^{-1}) is significantly different from zero, suggesting that the enthalpy of the conversion of the enzyme molecule itself from the low- to the high-temperature states is substantial and positive. In fact, if a decrease in the ground-state enthalpy of the enzyme upon entering the low-temperature state were to increase $\Delta H^{\ddagger}$ (which represents the difference in enthalpies of the ground and excited states) by a corresponding amount ($\simeq$ 10 kcal mole^{-1}), the overall rate of the ATPase reaction would be reduced by a factor of 2×10^{7}. Since a 10^{3}-fold decrease in the ATPase activity requires an increase in $\Delta G^{\ddagger}$ of only 4.1 kcal mole^{-1}, it is not difficult to see how a modest decrease in the ground-state enthalpy of the enzyme molecules in the low-temperature

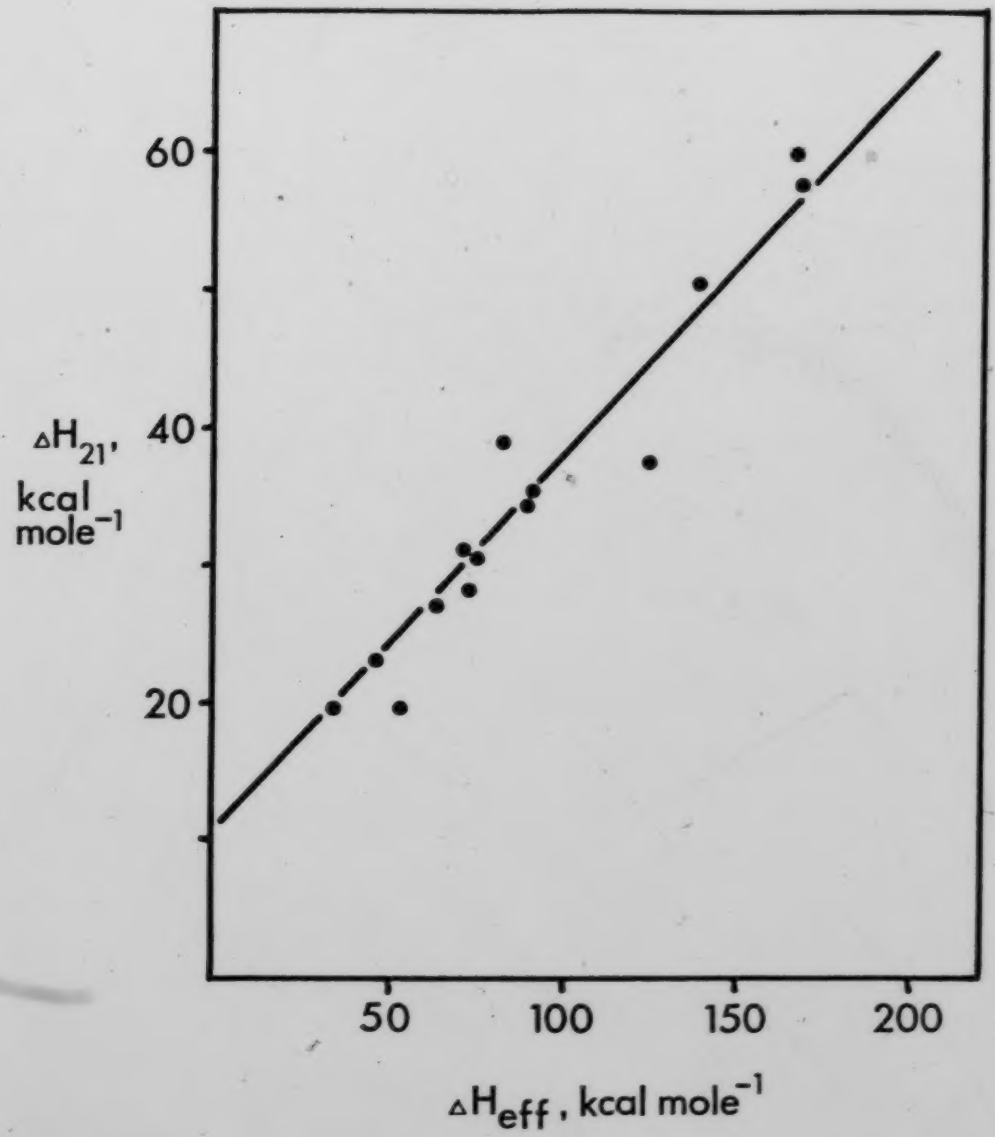

Figure 25. Correlation of the effective lipid phase transition enthalpy, ΔH_{eff}, with the effective enthalpy of transition of the ATPase from its low- to its high-temperature state, ΔH_{21}. These two parameters were determined in membrane preparations of various lipid fatty acid composition by analysis of differential thermograms and ATPase Arrhenius plots, respectively, as described in the text. The measured correlation coefficient is 0.90, and the coefficient of regression of ΔH_{21} on ΔH_{eff} is 0.27

state, if not fully compensated by a corresponding decrease in entropy, could lead to an effectively complete inactivation of the ATPase.

In summary, my quantitative analysis of the Arrhenius plots for the membrane (Na^+,Mg^{2+})-ATPase in various lipid environments suggests that the activity of the enzyme is regulated by the physical state, but not the detailed fatty acyl chain structure, of its boundary lipids, which undergo a phase change that is related to that of the bulk lipid phase but is of lower cooperativity. Marčelja (1976) has predicted on theoretical grounds that the boundary lipids associated with an integral membrane protein do in fact undergo a change in state that is 'driven' by the bulk lipid phase transition but is less cooperative, and experimental studies of the boundary lipids associated with the membrane protein glycophorin (Van Zoelen et al., 1978) and the membrane-active polypeptide antibiotic gramicidin A (Susi et al., 1979) have supported this prediction. The activity of the ATPase drops off greatly, to levels too low to quantify due to the presence of a contaminating ATP hydrolytic activity, when the boundary lipids are in their low-temperature state. This inactivation is probably explainable, at least in part, in terms of a decrease in the ground-state enthalpy of the enzyme molecule when the boundary lipids are in the low-temperature state, for such an enthalpy decrease could elevate $\Delta G^{\ddagger}$ if not exactly compensated by a decrease in the ground-state entropy of the enzyme.

Conclusions

The analysis of Arrhenius plot shapes discussed in the 'Theory' section above, and applied in the last section, offers a more useful basis for obtaining basic physical evidence about the behavior of a membrane

enzyme than do the analyses previously employed in membrane studies. The dissection of an Arrhenius plot into a series of straight-line regions is clearly artifical and can result in a loss of useful information about the true behavior of the system. Furthermore, the assumption that a 'break' in an Arrhenius plot thus dissected corresponds to the temperature of a significant thermotropic change in the state of a system is not universally justified. The concave-upward 'break' temperature in equation [7-7], for example, does not correspond to the low-temperature endpoint of a conformational or phase transition but is in fact a composite parameter at best, and may be wholly artifactual if a contaminating enzyme activity is present and emerges only when the major activity decreases to low levels. Furthermore, the upper 'break' predicted by equation [7-7] represents not the upper-temperature endpoint of a phase or conformational change but rather corresponds to the midpoint of the transition of the enzyme between two states. The two 'breaks' seen by a number of workers for Arrhenius plots of various membrane-bound enzyme or transport activities (Hesketh et al., 1976; Thilo et al., 1977) have usually been incorrectly interpreted as the endpoints of a thermotropic transition, and certain features of the Arrhenius plots which could furnish useful information (e.g., the slopes of the steeply sloping regions of the plots) have not been analyzed as thoroughly as might be possible by reference to a well-defined model. I regard the present work only as one stage in the development of more powerful strategies for the study of membrane enzymes through their kinetic properties. It is entirely possible that the conclusions I report here may prove to bear only a very tenuous relationship to the conclusions ultimately reached in future studies of this system, using more rigorous theories of the nature of lipid-

protein interactions and applying the knowledge gained from increasingly sophisticated studies of the physical properties of lipid-protein systems.

How well does the model derived from the experimental analysis described above agree with our basic understanding of the behavior of lipid-protein systems? If the rate-limiting step in the overall ATPase reaction involves a transient lateral displacement of lipid molecules accompanying a change in the conformation of the lipid-associated portion of the enzyme, then it is quite reasonable to assume that a major increase in the resistance of the lipids to displacement, such as would occur if the lipids entered the gel state, would greatly reduce the activity of the enzyme, as seems to be the case for the A. laidlawii B ATPase. Likewise, the finite and positive enthalpy change that the enzyme molecule appears to experience when the lipids around it enter the liquid-crystalline state (see Figure 25) is explainable in terms of a decreased strength of van der Waal's interactions between the protein and liquid-crystalline lipids, just as the strength of lateral association of the lipid molecules themselves decreases when the lipids enter the liquid-crystalline state. Finally, the apparent constancy of the enzyme activity's temperature dependence when the fatty acyl chains of the liquid-crystalline lipids around it are changed is consistent with the rather noteworthy insensitivity of many membrane enzyme activities in cultured mammalian cells to changes in the membrane lipid composition (Schroeder et al., 1976). Such insensitivity can be readily explained if one assumes that liquid-crystalline lipids in general provide an enzyme environment of suitably low microviscosity and high lateral compressibility that they do not provide significant resistance to a transient conforma-

tional change which occurs in the enzyme during its catalytic cycle. The activation enthalpy of the enzymic reaction will then depend primarily on factors intrinsic to the enzyme molecule itself, not on the exact composition of the lipid environment around it, so long as the lipids remain in the liquid-crystalline state. Further studies of the type described here, but using other enzyme systems, particularly those which can be readily reconstituted with pure synthetic lipids, could furnish a great deal of useful information regarding the generality of this aspect of the relationship of membrane enzyme activities to the properties of their lipid environments.

CHAPTER 8

CONCLUDING REMARKS

A Brief Synthesis

The work described in the foregoing chapters has ranged over a wide variety of topics, from lipid chemistry to microbial membrane energetics. It remains to relate the diverse types of studies discussed in this thesis to one another and to the goals outlined in Chapter 1. In this brief section, I will address this matter more directly than I have done in the preceding chapters.

As I noted in the introduction to this thesis, my overall concern in this work has been to develop more useful systems and more powerful analyses of the properties of membrane lipids, and their functional consequences, *in situ*. To this end, I have developed and extensively characterized the *A. laidlawii* B/avidin/Class I fatty acid system, which allows one to obtain substantial quantities of pure plasma membranes of homogeneous fatty acyl composition with minimal expenditure of effort and materials. While investigating the ability of different fatty acids to support growth of avidin-treated *A. laidlawii* B, I have made certain deductions from the results of various experiments which suggest that it is the physical and not the metabolic properties of a fatty acid that determine its growth-supporting ability in this organism. Studies of the compositional and lipid physical properties of fatty acid-homogeneous membranes have showed that changes occurring in membrane protein and head-group composition secondary to fatty acid changes are usually modest and are unlikely to qualitatively alter the behavior of the membrane lipids. By contrast, changes in the fatty acid composition of avidin-

treated A. laidlawii B can be used to vary the midpoint temperature and the sharpness of the membrane gel-to-liquid-crystalline transition temperature independently over a wide range of temperatures and transition widths, respectively. Therefore, as well as providing an excellent system for the evaluation of the 'functionalities' of various fatty acids when incorporated into membrane lipids, avidin-treated A. laidlawii B provides a very efficient means for greatly modifying the properties of the lipid phase in a biological membrane in vivo. To shed more light on the effects of varying fatty acid composition on membrane lipid properties, which clearly must be understood in order to interpret results obtained with membranes made homogeneous in fatty acids of widely varying structure, I have studied the thermotropic behavior of single pure lipids, the hydrated diacyl phosphatidylcholines, whose acyl chains were varied widely in both structure and chain length. My results with these pure lipids clearly demonstrate that the A. laidlawii B membrane lipids and the well-defined synthetic lipid system show entirely comparable changes in physical properties when the lipid fatty acyl composition is changed. It would appear, therefore, that the diacyl PC's derived from Class I fatty acids should provide a good model for the behavior of lipids in Class I fatty acid-homogeneous membranes of A. laidlawii B in cases where the latter system is still too heterogeneous for direct study by physical methods.

In the last two chapters, having evaluated the potential of the fatty acid-homogeneous A. laidlawii B membrane as an experimental system for studies of lipid 'functionalities' and physical properties, I turned to an analysis of the lipid modulation of a membrane enzyme activity, using some of the unique advantages of this system. A preliminary character-

ization of the enzyme raised some interesting questions regarding its function, which were answered to the extent that I can say with fair certainty that the (Na^+,Mg^{2+})-ATPase functions as a transmembrane ion pump essential for osmotic regulation. A systematic analysis of the temperature dependence of the enzyme activity in various fatty acid-homogeneous membranes was carried out in the light of physically realistic and analytically useful models of the enzyme behavior. Using my ability to widely vary the position of the phase transition midpoint in fatty acid-homogeneous membranes, I showed that the temperature dependence of the activity of the enzyme in a liquid-crystalline lipid environment is independent of the lipid fatty acyl composition, and that the downward-concave 'break' in ATPase Arrhenius plots is correlated with the occurrence of a membrane lipid phase transition. I then employed my ability to independently vary the sharpness of the lipid phase transition to provide support for a model in which a change in the state of the boundary lipids associated with the ATPase leads to an essentially complete loss of the enzyme's catalytic activity. This study of the A. laidlawii B membrane ATPase can, I feel, serve as a starting point for other analyses of the behavior of membrane enzymes in situ through an analysis of the temperature dependence of their activities. As well, this study demonstrates that the production of fatty acid-homogeneous membranes in A. laidlawii B brings some new and valuable types of experiments within the realm of possibility for the first time.

Finally, I wish to relate this work to the objectives outlined in the Introduction. As I hope the preceding survey of my work has made clear, while this study has not made use of most of the powerful and sophisticated physical methods for the study of membranes that I des-

cribed in Chapter 1, it has laid the foundation for the proper application of such methods to fatty acid-homogeneous membranes of A. laidlawii B and has provided some indication of the remaining limitations of the system for physical studies. As well, in this study I have demonstrated that the diacyl PC's generally should provide a very useful model system from which results may be derived for application to the A. laidlawii B system if the latter is too complex to be approached directly. At this time, Drs. Eric Oldfield (Urbana, Illinois) and Ian C.P. Smith (Ottawa, Ontario) are studying fatty acid-homogeneous membranes of A. laidlawii B by deuterium magnetic resonance, and our laboratory, in collaboration with that of Dr. Brian Sykes of this department, is beginning to study such membranes by ^{19}F-NMR, using specifically fluorinated fatty acids incorporated into membrane lipids as probes. Dr. Donald Engelman (New Haven, Connecticut) is currently carrying out X-ray diffraction studies of fatty acid-homogeneous A. laidlawii B membranes to extend the results derived from earlier studies with this organism (Engelman, 1971). It seems fair to predict that a number of novel and exciting results will be obtained through the use of this system, in conjunction with a variety of biochemical and physical techniques, over the next few years.

Three Important Questions

As I have devoted some one hundred and fifty pages to characterizing and evaluating the 'nuts and bolts' aspects of the fatty acid-homogeneous A. laidlawii B membrane as a valuable model system, it may seem rather foolish to raise more general questions about the utility and significance of the system at this point in my thesis. Nonetheless, I feel it necessary to briefly consider three questions which deserved to be

asked, and answered, about this system.

First, what are the significant advantages of this system that previously available systems do not offer? The greatest advantage of the fatty acid-homogeneous A. laidlawii B membrane is that the lipid composition can be greatly simplified, from hundreds of molecular species to only five significant glycerolipid species whose acyl chain structure can be varied over a wide range. Membranes of near-homogeneous lipid fatty acyl composition have been produced in E. coli (Baldassare et al., 1976, 1977), but only with a few fatty acids, all of which give membrane lipid phase transitions below 0°C, and in Mycoplasma strain Y (Rodwell, 1968; Rodwell and Peterson, 1971), which, however, can only grow in the presence of large amounts of membrane sterols, which broaden and reduce the enthalpy of the membrane lipid phase transition (Ladbrooke and Chapman, 1969). Fatty acid-homogeneous A. laidlawii B membranes thus provide a better link between pure synthetic phospholipid/water systems and biological membranes than do either of the fatty acid-homogeneous systems just described. To my knowledge, strong control of the sharpness of the lipid phase transition in a biological membrane by direct lipid manipulation has not previously been possible in any membrane whose lipid phase transition lies in the physiological temperature range. The potential for control of this aspect of the lipid phase transition is thus a major new advantage of the A. laidlawii B/avidin system. This system offers one further new advantage that may be a great usefulness in physical studies, namely the potential to incorporate fatty acid probes, such as deuterated, fluorinated, ^{13}C-enriched or fluorescent fatty acids, to very high levels into the membrane lipids without any metabolic modifications of their acyl chains. It seems fair to conclude, therefore, that the

development of the A. laidlawii B/avidin system provides a significantly improved system for the study of membrane lipid properties and their functional consequences.

Secondly, how suitable is the fatty acid-homogeneous A. laidlawii B membrane as a model system from which extrapolations can be made to, e.g., mammalian cell membranes? Obviously, the A. laidlawii B membrane cannot be considered identical to a eukaryotic cell membrane. The former system contains no sterols and only low levels of carotenoids and is thus less desirable than are the Mycoplasma cell membranes as a model system for the study of sterol functions in membranes. The A. laidlawii B membrane has a lipid head-group composition that is quite different from that of animal cells. Nonetheless, the quite comparable thermotropic behavior of fatty acid-homogeneous A. laidlawii lipids and the corresponding diacyl PC's suggests that this difference in head-group composition does not seriously limit the usefulness of the A. laidlawii B membrane as a model system for the membranes of higher organisms. A more significant limitation of the A. laidlawii system is the fact that certain important functions of animal cell membranes, such as exo- and endocytosis, mediation of cell-cell communication, and the maintenance of a fixed distribution of membrane receptor molecules, have not been observed in A. laidlawii B. This fact will limit the usefulness of this system for studying membrane lipid effects on certain membrane functions which are important in animal cells. To take a more positive view, however, one can study the effects of membrane lipid physical properties on cell growth and homeostasis, membrane enzyme activities, membrane energetics and membrane transport, to take some examples, in fatty acid-homogeneous A. laidlawii B, and all of these membrane functions are of course very

important aspects of the function of animal cell membranes as well. The A. laidlawii B membrane certainly provides the basic elements required for a wide variety of studies of membrane functions which are equally relevant to microbial and to mammalian cell membranes.

The final important question that I wish to address is the following: How strongly can experimentally realizable changes in the physical properties of membrane lipids affect the biological functions of the membrane? It is obviously of little value to make use of the A. laidlawii B/avidin system for studies of membrane lipid properties if the functional consequences of changes in these properties are minimal. Some recent studies have suggested that membrane 'fluidity' is not quite the all-important determinant of membrane function that it was sometimes thought to be in the early 1970's (Sinensky, 1974). Thus Mohan Das and Weeks (1979) have reported that the inhibition of slime mold differentiation caused by polyunsaturated fatty acid enrichment in membrane lipids is not accompanied by any change in membrane fluidity as detected by fatty acid spin-label ESR. Moya et al. (1979) and Axelrod et al. (1978) have recently reported that major changes in the membrane lipid fatty acid composition of cultured mammalian cells have no effect on cell-cell adhesion and on the rate of lateral diffusion of the acetylcholine receptor within the membrane, respectively. My finding that a wide variety of Class I fatty acids support good growth of A. laidlawii B in the presence of avidin, while giving widely varying phase transition temperatures when incorporated to homogeneity into the membrane lipids, certainly suggests that membrane lipid 'fluidity' need not be kept absolutely constant in order to support normal membrane function. In spite of these reservations, the literature contains enough well-documented examples of cases where a con-

trolled change in membrane lipid composition significantly alters one or more important functions of the membrane (Linden and Fox, 1973; Cronan and Gelmann, 1975; Baldassare et al., 1977; Horwitz et al., 1978, Engelhard et al., 1978) that it would be quite rash to say that lipid physical properties are wholly irrelevant to membrane function. In view of the foregoing observations, I would suggest that a great deal of work is needed to identify both what types of membrane-related processes are most susceptible to modulation by the physical properties of the membrane lipids, and what specific aspects of the lipid physical properties (lateral mobility? lateral compressibility? rate of acyl chain reorientation?) are most important in causing such modulation of membrane function. The fatty acid-homogeneous A. laidlawii B membrane system should prove to be of considerable value as an experimental system in which to seek answers to both of these questions.

Further Questions Raised by This Work

In this section, I wish to discuss a few questions which have been raised by the results discussed in this thesis, and some possible experimental approaches which could provide answers to them. Since my studies have been chiefly intended to provide better experimental systems and approaches for membrane research, a more general discussion of 'suggestions for future research' could rapidly balloon into a chapter of its own, and for this reason I shall refrain from a broader discussion of this type.

The first notable question raised by my results is that of the physical basis of the inhibition of growth of A. laidlawii B by 'hyperfluidizing' fatty acids when the cells are treated with avidin. We are

currently examining a variety of properties, including the (Na^+,Mg^{2+})-ATPase activity, the nonelectrolyte permeability and the osmotic fragility, of membranes made homogeneous in a variety of fatty acids in order to answer this question. Our preliminary results suggest that the integrity of the membrane permeability barrier is the most profoundly affected membrane property when progressively more 'fluidizing' fatty acids are incorporated into the cell membrane. A second interesting question arising from my work is that of the arrangement of the acyl chains (particularly the degree of chain tilt) in lipid-water systems with lipid acyl chains of varying structures. This question could be dealt with, at least in a general way, by X-ray or electron diffraction experiments (Hui, 1976; Brady and Fein, 1977) on oriented lipid bilayers, and if significant differences are found for lipids with different types of acyl chains, it would be interesting to see if such differences might have significant consequences for such membrane lipid properties as lateral diffusion rates or transbilayer permeation of solutes. A third question, arising from the work described in Chapter 6, concerns the exact role of the membrane (Na^+,Mg^{2+})-ATPase in the transport of such ions as H^+ and K^+ in A. laidlawii B. This problem, unfortunately, will probably be ultimately resolved only when the ATPase can be reconstituted into lipid vesicles in the absence of other proteins which could exhibit ion antiporter activity. Failing this, it would be useful to find a specific inhibitor of the ATPase and then to monitor the effect of this inhibitor on the transport of K^+ (by tracer analysis or by the use of an ion-selective electrode) and of H^+ (by using a fluorescent or radiolabeled molecule to monitor the pH difference across the membrane (Azzi and Montecucco, 1976)). Finally, it would be desirable to obtain direct physical confir-

mation of the model for lipid modulation of the A. laidlawii B membrane ATPase activity that I proposed in Chapter 7 on the basis of Arrhenius plot analyses. This could be accomplished if the enzyme were isolated and reconstituted with pure phospholipids whose thermotropic behavior could be monitored by high-sensitivity differential scanning calorimetry (HSDSC) while the temperature dependence of the enzyme activity in the reconstituted complex was analyzed in terms of the theory discussed in Chapter 7. If low levels of lipid were used in the reconstitution of the enzyme, the ratio of free to protein-bound lipid could be decreased to the extent that a broad phase transition in the latter lipid pool, driven by a sharper transition in the former, might be detectable by HSDSC if such a transition does occur, as my theory would predict. Work in our laboratory is in progress toward the goal of isolating the ATPase in an active form from the membrane, and some very preliminary results are encouraging. However, a good deal of work remains before we will be able to obtain substantial amounts of a pure ATPase preparation reconstituted with a synthetic phospholipid species.

REFERENCES

Abrahamsson, S., Ställberg-Stenhagen, S. and Stenhagen, E. (1963), Prog. Chem. Fats and Other Lipids 7, 1.

Altenbern, R.A. (1977), Antimicrob. Ag. Chemother. 11, 574.

Ames, D.E. and Covell, A.N. (1963), J. Chem. Soc. (London), 775.

Andrich, M.P. and Vanderkooi, J.M. (1976), Biochemistry 15, 1257.

Astin, A.M. and Haslam, J.M. (1977), Biochem. J. 166, 287.

Astin, A.M., Haslam, J.M. and Woods, R.A. (1977), Biochem. J. 166, 275.

Atkinson, A., Gatenby, A.D. and Lowe, A.O. (1973), Biochim. Biophys. Acta 320, 195.

Axelrod, D., Koppel, D.E., Schlessinger, J., Elson, E. and Webb, W.W. (1976), Biophys. J. 16, 1055.

Axelrod, D., Wight, A., Webb, W.W. and Horwitz, A. (1978), Biochemistry 17, 3604.

Azzi, A. and Montecucco, C. (1976), J. Bioenerg. Biomemb. 8, 257.

Baldassare, J.J., Rhinehart, K.B. and Silbert, D.F. (1976), Biochemistry 15, 2986.

Baldassare, J.J., Brenckle, G.M., Hoffman, M. and Silbert, D.F. (1977), J. Biol. Chem. 252, 8797.

Bangham, A.D., Hill, M.W. and Miller, N.G.A. (1974), in Methods in Membrane Biology, v. 1, E.D. Korn, Ed., Plenum Press, New York, p. 1.

Barton, P.G. and Gunstone, F.D. (1975), J. Biol. Chem. 248, 4470.

Bergelson, L.D. and Barsukov, L.I. (1977), Science 197, 224.

Bevers, E.M., Singal, S.A., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1977), Biochemistry 16, 1290.

Bevers, E.M., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1978), Eur. J. Biochem. 84, 35.

Bligh, E.J. and Dyer, W.J. (1959), Can. J. Biochem. Physiol. 37, 911.

Boggs, J.M., Moscarello, M.A. and Papahadjopoulos, D. (1977a), Biochemistry 16, 5420.

Boggs, J.M., Wood, D.D., Moscarello, M.A. and Papahadjopoulos, D. (1977b), Biochemistry 16, 2325.

Brady, G.W. and Fein, D.B. (1977), Biochim. Biophys. Acta 464, 249.

Bretscher, M.C. (1973), Science 181, 622.

Butler, K.W., Johnson, K.G. and Smith, I.C.P. (1978), Arch. Biochem. Biophys. 191, 289.

Buttke, T.M. and Ingram, L.O. (1978), Biochemistry 17, 5282.

Cadenhead, D.A. and Müller-Landau, F. (1975), Adv. Chem. 144, 294.

Cadenhead, D.A., Kellner, B.M.J., Jacobson, P. and Papahadjopoulos, D. (1977), Biochemistry 16, 5386.

Capaldi, R.A. (1977), in Membrane Proteins and their Interations with Lipids, R.A. Capaldi, ed., Marcel Dekker, New York, p. 1.

Chapman, D. (1965), The Structure of LIpids, John Wiley and Sons, New York.

Chen, R.F. (1967), J. Biol. Chem. 242, 1973.

Chen, H.W., Heineger, H.-J. and Kandutsch, A.A. (1978), J. Biol. Chem. 253, 3180.

Chen, L.A., Dale, R.E., Roth, S. and Brand, L. (1977), J. Biol. Chem. 252, 2163.

Cho, H.W. and Morowitz, H.J. (1969), Biochim. Biophys. Acta 183, 295.

Christensen, H.N. (1975), Biological Transport, 2nd ed., W.A. Benjamin, London.

Christiansson, A. and Wieslander, Å (1978), Eur. J. Biochem. 85, 65.

Christie, W.W. (1973), Lipid Analysis, Pergamon Press, Oxford.

Christie, W.W., Gunstone, F.D., Ismail, I.A. and Wade, L. (1968), Chem. Phys. Lipids 2, 196.

Clarke, S. (1977), Biochem. Biophys. Res. Commun. 79, 46.

Cronan, J.E., Jr. and Gelmann, E.P. (1975), Bacteriol. Rev. 39, 232.

Cullis, P.R. and De Kruijff, B. (1976), Biochim. Biophys. Acta 507, 207.

Cullis, P.R. and Hope, M.J. (1978), Nature 271, 672.

Dale, R.E., Chen, L.A. and Brand, L. (1977), J. Biol. Chem. 252, 7500.

Davis, M.-T. and Silbert, D.F. (1974), Biochim. Biophys. Acta 363, 1.

De Gier, J., Mandersloot, J.G. and Van Deenen, L.L.M. (1968), Biochim. Biophys. Acta 150, 666.

De Kruijff, B., Demel, R.A., Slotboom, A.J., Van Deenen, L.L.M. and Rosenthal, A.F. (1973), Biochim. Biophys. Acta 307, 1.

De Kruyff, B., Van Dijck, P.W.M., Goldbach, R.W., Demel, R.A. and Van Deenen, L.L.M. (1973), Biochim. Biophys. Acta 330, 269.

Demel, R.A., Jansen, J.W.C.M., Van Dijck, P.W.M. and Van Deenen, L.L.M. (1977), Biochim. Biophys. Acta 465, 1.

De Pont, J.J.H.H.M., Van Prooijen-Van Eeden, A. and Bonting, S.L. (1978), Biochim. Biophys. Acta 508, 464.

Deuticke, B. and Ruska, C. (1976), Biochim. Biophys. Acta 433, 638.

Dockter, M.E., Trumble, W.R. and Magnuson, J.A. (1978), Proc. Nat. Acad. Sci. U.S.A. 75, 1319.

Dufourq, J., Bernon, R. and Lussan, C. (1976), Biochim. Biophys. Acta 433, 252.

Edidin, M. (1974), Ann. Rev. Biophys. Bioeng. 3, 179.

Engelhard, V.H., Glaser, M. and Storm, D.R. (1978), Biochemistry 17, 3191.

Engelman, D.M. (1971), J. Mol. Biol. 58, 153.

Enuch, H.G., Fleming, P.J. and Strittmater, P. (1977), J. Biol. Chem. 252, 5656.

Findlay, E.J. and Barton, P.G. (1978), Biochemistry 17, 2400.

Folch, J., Lees, M. and Stanley, G.H. (1957), J. Biol. Chem. 226, 497.

Gaber, B.P. and Peticolas, W.L. (1977), Biochim. Biophys. Acta 465, 260.

Gaber, B.P., Yager, P. and Peticolas, W.L. (1978a), Biophys. J. 21, 161.

Gaber, B.P., Yager, P. and Peticolas, W.L. (1978b), Biophys. J. 22, 191.

Gatt, S. and Bartfai, T. (1977), Biochim. Biophys. Acta 488, 1.

Gent, M.P.N. and Ho, C. (1978), Biochemistry 17, 3023.

Gilman, N. and Holland, B. (1974), Synth. Commun. 4, 199.

Goldberg, I., Walker, J.R. and Bloch, K. (1973), Antimicrob. Ag. Chemother. 3, 549.

Guidotti, G. (1977), J. Supramol. Struc. 7, 489.

Gulik-Krzywicki, T. (1975), Biochim. Biophys. Acta 415, 1.

Gunstone, F.D. (1967), An Introduction to the Chemistry and Biochemistry of Fatty Acids and their Glycerides, Chapman and Hall, London.

Gunstone, F.D. and Perera, B.S. (1973), Chem. Phys. Lipids 10, 303.

Harold, F.M. (1977), Curr. Topics Bioenerg. 6, 83.

Hartree, E.T. (1972), Anal. Biochem. 48, 422.

Hatten, M.E., Horwitz, A.F. and Burger, M.M. (1977), Exp. Cell Res. 107, 31.

Hauser, H. and Barratt, M.D. (1973), Biochem. Biophys. Res. Commun. 53, 399.

Hauser, H., Finer, E.G. and Darke, A. (1977), Biochem. Biophys. Res. Commun 76, 267.

Hawrot, E. and Kennedy, E.P. (1978), J. Biol. Chem. 253, 8213.

Henderson, R. and Unwin, P.N.T. (1975), Nature 257, 28.

Hesketh, T.R., Smith, G.A., Houslay, M.D., McGill, K.A., Birdsall, N.J.M., Metcalfe, J.C. and Warren, G.B. (1976), Biochemistry 15, 4145.

Hilden, S. and Hokin, L. (1976), Biochem. Biophys. Res. Commun. 69, 521.

Hofman, K., Jucker, O., Miller, W.R., Young, A.C., Jr. and Tausig, F. (1954), J. Am. Chem. Soc. 76, 1799.

Hofman, K., Orochena, S.F. and Yoho, C.W. (1957), J. Am. Chem. Soc. 79, 3608.

Horwitz, A.F., Wight, A., Ludwig, P. and Cornell, R. (1978), J. Cell Biol. 77, 334.

Houslay, M.D., Hesketh, T.R., Smith, G.A., Warren, G.B. and Metcalfe, J.C. (1976), Biochim. Biophys. Acta 436, 495.

Hubbell, W.L. and McConnell, H.M. (1971), J. Am. Chem. Soc. 93, 314.

Hui, S.W. (1976), Chem. Phys. Lipids 16, 9.

Jackson, M.B. and Cronan, J.E., Jr. (1978), Biochim. Biophys. Acta 512, 472.

Jacobson, K. and Papahadjopoulos, D. (1976), Biophys. J. 16, 549.

James, R. and Branton, D. (1973), Biochim. Biophys. Acta 323, 378.

James, T.L. (1975), Nuclear Magnetic Resonance in Biochemistry: Principles and Applications, Academic Press, New York.

Janiak, M.J., Small, D.M. and Shipley, G.G. (1976), Biochemistry 15, 4575.

Jinks, D.C., Silvius, J.R. and McElhaney, R.N. (1978), J. Bacteriol, 136, 1027.

Johnson, R.H., Eyring, H. and Stover, B.J. (1974), The Theory of Rate Processes in Biology and Medicine, John Wiley and Sons, New York.

Jost, P.C. and Griffith, O.H. (1978), in Biomolecular Structure and Function, P.F. Agris, R.N. Loeppky and B.D. Sykes, eds., Academic Press, New York. p. 25.

Jost, P.C., Griffith, O.H., Capaldi, R.A. and Vanderkooi, G. (1973), Proc. Nat. Acad. Sci. U.S.A. 70, 480.

Jost, P.C., Nadakavukaren, K.K. and Griffith, O.H. (1977), Biochemistry 16, 3110.

Kahane, I., Ne'eman, Z. and Razin, S. (1973), J. Bacteriol. 113, 666.

Kahane, I. and Razin, S. (1977), Biochim. Biophys. Acta 471, 32.

Kaneda, T. (1977), Microbiol. Rev. 41, 391.

Khan, N.A., Deatherage, F.E. and Brown, J.B. (1963), Org. Synth. Coll. 4, 851.

Kinosita, K., Jr., Kawato, S. and Ikegami, A. (1977), Biophys. J. 20, 289.

Koppel, D.E., Axelrod, D., Schlessinger, J., Elson, E.L. and Webb, W.W. (1976), Biophys. J. 16, 1315.

Koyama, Y., Toda, S. and Kyogoku, Y. (1977), Chem. Phys. Lipids 19, 74.

Ladbrooke, B.D. and Chapman, D. (1969), Chem. Phys. Lipids 3, 304.

Ladbrooke, B.D., Williams, R.M. and Chapman, D. (1968), Biochim. Biophys. Acta 150, 333.

Larsson, K. (1977), Chem. Phys. Lipids 20, 225.

Lee, A.G. (1977a), Biochim. Biophys. Acta 472, 237.

Lee, A.G. (1977b), Biochim. Biophys. Acta 472, 285.

Lee, A.G., Birdsall, N.J.M. and Metcalfe, J.C. (1974), in Methods in Membrane Biology, v. 2, E.D. Korn, ed., Plenum Press, New York, p. 1.

Lee, A.G., Birdsall, N.J.M., Metcalfe, J.C., Warren, G.B. and Roberts, G.C.K. (1976), Proc. Royal Soc. London B, 253.

Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976), Biochemistry 15, 4521.

Linden, C.D. and Fox, C.F. (1975), Accounts Chem. Res. 8, 321.

Lowry, O.H., Roseborough, N.J., Farr, A.L. and Randall, R.J. (1951), J. Biol. Chem. 193, 265.

Lynn, R.J. (1960), Ann. N.Y. Acad. Sci. 79, 538.

Mabrey, S. and Sturtevant, J.M. (1976), Proc. Nat. Acad. Sci. U.S.A. 73, 3862.

Malkin, T. (1952), Prog. Chem. Fats and Other Lipids 1, 1.

Mandersloot, J.G., Reman, F.C., Van Deenen, L.L.M. and De Gier, J. (1975), Biochim. Biophys. Acta 382, 22.

Mandersloot, J.G., Roelofsen, B. and De Gier, J. (1978), Biochim. Biophys. Acta 508, 478.

Maniloff, J. (1969), Microbios 2, 125.

Marčelja, S. (1976), Biochim. Biophys. Acta 455, 1.

Mason, R.P. and Polnaszek, C.F. (1978), Biochemistry 17, 1758.

McElhaney, R.N. (1974a), J. Mol. Biol. 84, 145.

McElhaney, R.N. (1974b), J. Supramol. Struc. 2, 617.

McElhaney, R.N. and Tourtellotte, M.E. (1969), Science 164, 433.

McElhaney, R.N. and Tourtellotte, M.E. (1970), Biochim. Biophys. Acta 202, 120.

McElhaney, R.N., De Gier, J. and Van Deenen, L.L.M. (1970), Biochim. Biophys. Acta 219, 245.

Melchior, D.L. and Steim, J.M. (1976), Ann. Rev. Biophys. Bioeng. 5, 205.

Mendelsohn, R. and Maisano, J. (1978), Biochim. Biophys. Acta 506, 192.

Mohan Das, D.V. and Weeks, G. (1979), Exp. Cell Res. 118, 237.

Moya, F., Silbert, D.F. and Glaser, M. (1979), Biochim. Biophys. Acta 550, 485.

Niederberger, W. and Seelig, J. (1976), J. Am. Chem. Soc. 98, 3704.

Nozaki, Y., Reynolds, J.A. and Tanford, C. (1978), Biochemistry 17, 1239.

Nozawa, Y. (1973), J. Biochem. (Tokyo) 74, 1157.

Ohno, T., Awaya, J., Kesado, T., Nomura, S. and Ōmura, S. (1974), Antimicrob. Ag. Chemother. 6, 387.

Ohta, A. and Shibuya, I. (1977), J. Bacteriol. 132, 434.

Ōmura, S. (1976), Bacteriol. Rev. 40, 681.

Papahadjopoulos, D. (1973), in Form and Function of Phospholipids, 2nd ed., G.B. Ansell, R.M.C. Dawson and J.N. Hawthorne, eds., Elsevier Scientific Publishing Co., Amsterdam, p. 143.

Papahadjopoulos, D., Vail, W.J., Jacobson, K. and Poste, G. (1975), Biochim. Biophys. Acta 394, 483.

Patel, K.M. and Sparrow, J.T. (1979), Synth. Commun. 9, in press.

Patel, K.M., Morrisett, J.D. and Sparrow, J.T. (1979), J. Lipid Res., in press.

Pisetsky, D. and Terry, T.M. (1972), Biochim. Biophys. Acta 274, 95.

Pluschke, G., Hirota, Y. and Overath, P. (1978), J. Biol. Chem. 253, 5048.

Pollack, J.D., Razin, S. and Cleverdon, R.C. (1965a), J. Bacteriol. 90, 617.

Pollack, J.D., Razin, S., Pollack, M.E. and Cleverdon, R.C. (1965b), Life Sci. 4, 973.

Poste, G. and Papahadjopoulos, D. (1976), Proc. Nat. Acad. Sci. U.S.A. 73, 1603.

Raetz, C. and Foulds, J. (1977), J. Biol. Chem. 252, 5911.

Raison, J.K. (1973), Bioenergetics 4, 285.

Rand, R.P., Chapman, D. and Larsson, K. (1975), Biophys. J. 15, 1117.

Rodin, B. (1970), Calculus with Analytic Geometry, Prentice-Hall Inc., Eaglewood Cliffs, N.J.

Rodwell, A.W. (1967), Ann. N.Y. Acad. Sci. 143, 88.

Rodwell, A.W. (1968), Science 160, 1350.

Rodwell, A.W. and Peterson, J.E. (1971), J. Gen. Microbiol. 68, 173.

Roelofsen, B. and Zwaal, R.F.A. (1976), in Methods in Membrane Biology, v. 7, E.D. Korn, Ed., Plenum Press, New York, p. 147.

Ronquist, G. (1975), in Biological Transport, 2nd ed., H.N. Christensen, ed., W.A. Benjamin, Inc., London, p. 272.

Rothman, J.E. and Lenard, J. (1977), Science 195, 743.

Rottem, S. and Barile, M.F. (1976), Antimicrob. Ag. Chemother. 9, 301.

Rottem, S. and Razin, S. (1966), J. Bacteriol. 92, 714.

Rottem, S. and Samuni, A. (1973), Biochim. Biophys. Acta 298, 32.

Rottem, S., Hubbell, W.L., Hayflick, L. and McConnell, H.M. (1970), Biochim. Biophys. Acta 219, 104.

Saito, Y. and McElhaney, R.N. (1977), J. Bacteriol. 132, 485.

Saito, Y. and McElhaney, R.N. (1978), Biochim. Biophys. Acta 529, 224.

Saito, Y., Silvius, J.R. and McElhaney, R.N. (1977a), J. Bacteriol. 132, 497.

Saito, Y., Silvius, J.R. and McElhaney, R.N. (1977b), Arch. Biochem. Biophys. 182, 443.

Saito, Y., Silvius, J.R. and McElhaney, R.N. (1978), J. Bacteriol. 133, 66.

Salem, L. (1962), Can. J. Biochem. Physiol. 40, 1287.

Sandermann, H., Jr. (1978), Biochim. Biophys. Acta 515, 209.

Schroeder, F., Perlmutter, J.F., Glaser, M. and Vagelos, P.R. (1976), J. Biol. Chem. 251, 5015.

Seelig, A. and Seelig, J. (1974), Biochemistry 13, 4839.

Seelig, A. and Seelig, J. (1977), Biochemistry 16, 45.

Seelig, J. and Browning, J.L. (1978), FEBS Lett. 92, 41.

Seelig, J. and Waespe-Šarčevič, N. (1978), Biochemistry 17, 3310.

Seeman, P. (1972), Pharmacol. Rev. 24, 583.

Selinger, Z. and Lapidot, Y. (1966), J. Lipid Res. 7, 174.

Shinitzky, M. and Barenholz, Y. (1978), Biochim. Biophys. Acta 515, 367.

Silbert, D.F. (1975), Ann. Rev. Biochem. 44, 315.

Silvius, J.R., Saito, Y. and McElhaney, R.N. (1977), Arch. Biochem. Biophys. 182, 455.

Silvius, J.R. and McElhaney, R.N. (1978), Can. J. Biochem. 56, 462.

Silvius, J.R. and McElhaney, R.N. (1979a), Chem. Phys. Lipids 24, in press.

Silvius, J.R. and McElhaney, R.N. (1979b), Chem. Phys. Lipids, in press.

Silvius, J.R. and McElhaney, R.N. (1979c), J. Theor. Biol., submitted for publication.

Silvius, J.R. and McElhaney, R.N. (1979d), Chem. Phys. Lipids, submitted for publication.

Sinensky, M. (1974), Proc. Nat. Acad. Sci. U.S.A. 71, 522.

Singer, S.J. (1974), Ann. Rev. Biochem. 43, 805.

Singer, S.J. (1977), J. Supramol. Struc. 6, 313.

Singer, S.J. and Nicolson, G.L. (1972), Science 175, 720.

Sklar, L.A., Hudson, B.S. and Simoni, R.D. (1975), Proc. Nat. Acad. Sci. U.S.A. 72, 1649.

Sklar, L.A., Hudson, B.S. and Simoni, R.D. (1976), J. Supramol. Struc. 4, 449.

Smith, P.F. (1971), The Biology of Mycoplasmas, Academic Press, New York.

Smith, P.F. and Henrickson, C.V. (1966), J. Bacteriol. 91, 1854.

Smith, B.A. and McConnell, H.M. (1978), Proc. Nat. Acad. Sci. U.S.A. 75, 2759.

Smothers, W.J. and Chiang, Y. (1966), Handbook of Differential Thermal Analysis, Chemical Publishing Co., New York.

Steim, J.M., Tourtellotte, M.E., Reinert, J.C., McElhaney, R.N. and Rader, R.L. (1969), Proc. Nat. Acad. Sci. U.S.A. 63, 104.

Sturtevant, J.N. and Mateo, P.L. (1978), Proc. Nat. Acad. Sci. U.S.A. 75, 2584.

Susi, H., Sampugna, J., Hampson, J.W. and Ard, J.S. (1979), Biochemistry 18, 297.

Tardieu, A., Luzzati, V. and Reman, F.C. (1973), J. Mol. Biol. 75, 711.

Tarshis, M.A. and Kapitanov, A.B. (1978), FEBS Lett. 89, 73.

Thilo, L., Träuble, H. and Overath, P. (1977), Biochemistry 16, 1283.

Thulborn, K.R., Treloar, F.E. and Sawyer, W.H. (1978), Biochem. Biophys. Res. Commun. 81, 42.

Tourtellotte, M.E., Branton, D. and Keith, A.D. (1970), Proc. Nat. Acad. Sci. U.S.A. 66, 909.

Träuble, H. (1971), J. Memb. Biol. 4, 193.

Träuble, H. and Eibl, H. (1974), Proc. Nat. Acad. Sci. U.S.A. 71, 214.

Tsuchiya, T. and Rosen, B.P. (1975), J. Biol. Chem. 250, 8409.

Unwin, P.N.T. and Henderson, R. (1975), J. Mol. Biol. 94, 425.

Van Dijck, P.W.M., De Kruijff, B., Van Deenen, L.L.M., De Gier, J. and Demel, R.A. (1976), Biochim. Biophys. Acta 455, 576.

Van Dijck, P.W.M., De Kruijff, B., Verkleij, A.J., Van Deenen, L.L.M. and De Gier, J. (1978), Biochim. Biophys. Acta 512, 84.

Van Zoelen, E.J.J., Van der Neut-kok, E.C.M., De Gier, J. and Van Deenen, L.L.M. (1975), Biochim. Biophys. Acta 394, 463.

Van Zoelen, E.J.J., Van Dijck, P.W.M., De Kruijff, B., Verkleij, A.J. and Van Deenen, L.L.M. (1978), Biochim. Biophys. Acta 514, 9.

Verkleij, A.J. and Ververgaert, P.H.J.T. (1975), Ann. Rev. Phys. Chem. 26, 101.

Verkleij, A.J., Ververgaert, P.H.J.T., Van Deenen, L.L.M. and Elbers, P.F. (1972), Biochim. Biophys. Acta 288, 326.

Verma, S.P. and Wallach, D.F.H. (1976), Biochim. Biophys. Acta 436, 307.

Verma, S.P., Wallach, D.F.H. and Schmidt-Ullrich, R. (1975), Biochim. Biophys. Acta 394, 633.

Vik, S.B. and Capaldi, R.A. (1977), Biochemistry 16, 5755.

Volpe, J.J. and Vagelos, P.R. (1976), Physiol. Rev. 56, 339.

Vorbeck, M.L. and Marinetti, G.V. (1965), J. Lipid Res. 6, 3.

Wakil, S.J. (1970), in Lipid Metabolism, S.J. Wakil, ed., Academic Press, New York, p. 1.

Wallace, B.A. and Engelman, D.M. (1978), Biochim. Biophys. Acta 508, 431.

Weber, K. and Osborn, M. (1969), J. Biol. Chem. 244, 4406.

Wieslander, Å. and Rilfors, L. (1977), Biochim. Biophys. Acta 466, 336.

Wieslander, Å, Ulmius, J., Lindblom, G. and Fontell, K. (1978), Biochim. Biophys. Acta 512, 241.

Wilkinson, G.N. (1961), Biochem. J. 80, 324.

Yeagle, P.L., Hutton, W.C., Huang, C.-h. and Martin, R.B. (1977), Biochemistry 16, 4344.

Yellin, N. and Levin, I.W. (1977), Biochemistry 16, 642.

Zahler, W.L. (1974), Methods Enzymol. 32B, 70.

Zilversmit, D.B. and Hughes, M.E. (1976), in Methods in Membrane Biology, v. 7, E.D. Korn, ed., Plenum Press, New York, p. 211.

Appendix 1

On Fatty Acid Nomenclature

In view of the very powerful methods now available for determining the structures of small molecules such as fatty acids, one could persuasively argue that the trivial nomenclature of fatty acids should be wholly abandoned, there no longer being any need to name all-*cis*-5,8,11,-14-eicosatetraenoic acid 'arachidonic acid' because it was first obtained from peanuts or (+)-ℓ-10-methyloctadecanoic acid 'tuberculostearic acid' because it was first isolated from a tubercule bacillus. However, all signs from the current biochemical literature suggest that the trivial nomenclature continues to be far more popular than the more systematic but colder and more formidable IUPAC system. Therefore, I have used the trivial nomenclature in this thesis wherever the trivial name for a fatty acid is widely used in the literature. This will, I hope, make the thesis more immediately comprehensible to lipid workers and avoid subjecting the non-expert to a disorienting shift in nomenclature when comparing this work with the published literature.

As an aid to understanding the trivial nomenclature, I have supplemented the trivial names of fatty acids with their standard shorthand symbols (in parentheses) the first time the former appear in a given section of the text. I have also used the shorthand symbolic nomenclature exclusively in most of the tables and figures. This latter nomenclature represents the complete structure of a fatty acid in a concise form, with the following elements:

(1) An initial number, giving the total number of carbons in the fatty acid molecule. This is followed by a colon, then by

(2) a number giving the total number of double bonds in the molecule, or the lower-case letters 'cp' if a cyclopropane ring is present. This will be followed, if necessary, by

(3) a lower-case letter or letters, as follows: 'i' for iso fatty acids ending in an isopropyl group, 'ai' for anteiso species ending in a 1-methylpropyl group, and 'c' or 't' if the double bond(s) or the cyclopropane ring has a cis- or a trans- configuration, respectively. If double bonds or cyclopropane rings are present, their positions are indicated by

(4) the symbol Δ, followed by superscript numbers giving the numbers of the lowest-numbered carbons in the acyl chain (numbering along the main chain from the carboxyl group) that are part of the double bonds or cyclopropane rings.

If the molecule is potentially optically active, its sign or configuration appears in front of the symbols just discussed. In this shorthand system, palmitic (hexadecanoic) acid is represented as 16:0, isopalmitic (14-methylpentadecanoic) acid as 16:0i, oleic (cis-9-octadecenoic) acid as 18:1cΔ^{9}, and lactobacillic (ℓ-cis-11,12-methyleneoctadecanoic) acid as ℓ 19:cp,cΔ^{11}.

Appendix 2

Calculation of the Fraction of di-B Glycerolipids in a Mixture of Lipids Whose Acyl Chain Composition Approaches a Binary Mixture of A and B

Assume that the overall scheme for production of diacyl glycerolipids GXY (where X and Y are the lipid acyl chains and G the lipid polar 'backbone') is as follows:

$$G \xrightarrow{\alpha[A]} GA \xrightarrow{a[A]} GAA$$
$$GA \xrightarrow{b[B]} GAB$$
$$G \xrightarrow{\beta[B]} GB \xrightarrow{a[A]} GBA$$
$$GB \xrightarrow{b[B]} GBB$$

where α, β, a and b are rate constants and [A] and [B] the effective concentrations of fatty acid species A and B at the site of complex lipid synthesis. If we then represent the fractions of GAA, GAB, etc., species in the total lipid products as AA, AB, etc., it can easily be shown that

$$\frac{a[A]}{b[B]} = \frac{AA}{AB} = \frac{BA}{BB}$$

and that

$$\frac{\alpha[A]}{\beta[B]} = \frac{AA + AB}{BA + BB} = \frac{AA(1 + b[B]/a[A])}{BA(1 + b[B]/a[A])} = \frac{AA}{BA}$$

where the third term is derived from the second by use of the first equation above. If we define (P_2/P_1) for a fatty acid as the amount of that species present at the 2-position of a diacylglycerolipid divided by the amount of that species present at the 1-position, we can write

$$\frac{(P_2/P_1)_B}{(P_2/P_1)_A} = \frac{(BB + AB)/(BB + BA)}{(BA + AA)/(AA + AB)} = \frac{\alpha b}{a\beta}$$

where the last term is derived by successive applications of the equations given above. As (P_2/P_1) can be determined experimentally (McElhaney and Tourtelotte, 1970; Saito et al., 1977; Saito and McElhaney, 1978), we can determine the right-hand term in the above set of identities from one set of experimental data, then apply it to another experiment in which [A] and [B] may be different. If the lipid fatty acid composition at least approximates a binary mixture, considerations of mass balance lead to the equation

$$\frac{\alpha b}{a\beta} \equiv A = \frac{(P_2/P_1)_B\,(1 + (P_2/P_1)_B - 2F_B)}{1 + (P_2/P_1)_B - 2(P_2/P_1)_B F_B}$$

where F_B is the fraction of B in the total lipid acyl chains. From this equation, we can determine $(P_2/P_1)_B$ as a function of F_B, according to the equation

$$(P_2/P_1)_B = \frac{(2F_B - 1)(1 - A) + ((1 - A)^2(1 - 2F_B)^2 + 4A)^{1/2}}{2}$$

and from this equation, the percentage of lipid GBB in the total lipid products, as follows:

$$\%\ \text{GBB} = \frac{(4)(100\%)\ F_B^{\,2}\ (P_2/P_1)_B}{(1 + (P_2/P_1)_B)^2}$$

In practice, I determined the constant A from previously published (P_2/P_1) data (given in the references listed above), then used this value to calculate $(P_2/P_1)_B$ under the conditions described in Chapter 3. (Since palmitate was used as the second fatty acid in my experiments and in the previously reported measurements of P_2/P_1, A should be the same in my experiments and the previous ones.) Once I had calculated $(P_2/P_1)_B$ for my experiments, I then calculated % GBB according to the above equation, obtaining the results given in the last column of Table 4.

Appendix 3

Analysis of Irreversible Enzyme Inhibition

Our starting point is the following model:

$$E + I \underset{K_I = K_{diss}}{\rightleftharpoons} E\cdot I \xrightarrow{k} EI \text{ (inactive)}$$

where E·I represents a reversibly formed, noncovalent complex of enzyme and inhibitor and EI is the inactive covalent enzyme-inhibitor complex. By analogy with Michaelis-Menten kinetics for the steady state,

$$(1) \qquad \frac{d(EI)}{dt} = \frac{k[E][I]}{[I]+K_I} = -\frac{dE}{dt}$$

and if K_I is very large or if the inhibitor is strongly diluted prior to assay of the enzyme activity, so that little or no E·I is present during the assay, we find that

$$(2) \qquad E(t) = E^o \exp\left(-\frac{k[I]t}{K_I + [I]}\right)$$

where E(t) is the residual enzyme activity after incubating for time t with the inhibitor, and E^o is the initial (uninhibited) activity. If E and E·I are not in rapid equilibrium, equations (1) and (2) are still valid, but K_I will not in general be equal to K_{diss} for the E·I complex. Successive logarithmic and reciprocal transformations of equation (2) give us the following equation:

$$(3) \qquad [\ln(E^o/E(t))]^{-1} = \left(\frac{K_I}{k}\right)\left(\frac{1}{[I]}\right) + \frac{1}{k}$$

The regression of the left-hand term on (1/[I]) will therefore be linear for this model, giving $-1/K_I$ as the x-intercept and 1/k as the y-intercept.

A nonlinear regression indicates the invalidity of one (or more) of the assumptions of the model described above. Three simple cases may be commonly observed:

(1) The EI complex is not wholly inactive, in which case the graph of $[\ln(E^{o}/E(t))]^{-1}$ vs. $1/[I]$ will be concave downward.

(2) More than one enzyme species of widely varying sensitivity to I may be present, in which case the graph will also be concave downward, or

(3) Inhibition by I may be positively cooperative, in which case the graph will be concave upward.